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1. Your reference	IS/BP5621479
2. Patent application number *(The Patent Office will fill in this part)*	9710962.3
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	CAMBRIDGE UNIVERSITY TECHNICAL SERVICES LIMITED THE OLD SCHOOLS CAMBRIDGE CB2 1TS
Patents ADP number *(if you know it)*	
If the applicant is a corporate body, give the country/state of its incorporation	ENGLAND
4. Title of the invention	POLYKETIDES AND THEIR SYNTHESIS
5. Name of your agent *(if you have one)*	MEWBURN ELLIS
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP
Patents ADP number *(if you know it)*	109006

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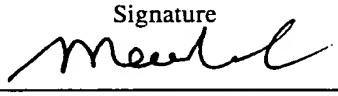
Signature 

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POLYKETIDES AND THEIR SYNTHESIS

The present invention relates to novel polyketides and methods and means for preparing them by recombinant synthesis. Polyketide biosynthetic genes or portions of them, which may be derived from different polyketide biosynthetic gene clusters are manipulated to allow the production of specific novel hybrid polyketides of predicted structure. The invention also relates to novel host-vector systems allowing increased levels of production of both natural and non-natural polyketides.

Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin and FK506. In particular, polyketides are abundantly produced by Streptomyces and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β-hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of

chain extension.

The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS) have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, avermectin and rapamycin (Figure 1), consists of a different set or "module" of enzymes for each cycle of polyketide chain extension (Figure 2)(Cortes, J. et al. Nature (1990) 348:176-178; Donadio, S. et al. Science (1991) 252:675-679; MacNeil, D. J. et al. Gene (1992), 115:119-125; Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843). Note: the term "natural module" as used herein refers to the set of contiguous domains, from a β-ketoacylsynthase ("KS") gene to the next acyl carrier protein ("ACP") gene, which accomplishes one cycle of polyketide chain extension. The term "combinatorial module" is used to refer to any group of contiguous domains (and domain parts), extending from a first point in a first natural module, to a second equivalent point in a second natural module. The first and second points will generally be in core domains which are present in all modules, ie both at equivalent points of respective KS, AT (acyl transferase) or ACP domains. The length of polyketide formed has been altered, in the case of erythromycin biosynthesis, by specific relocation using genetic engineering of the enzymatic domain of the

erythromycin-producing PKS that contains the chain-releasing thioesterase/cyclase activity (Cortes, J. et al. Science (1995) 268:1487-1489; Kao, C. M. et al. J. Am. Chem. Soc. (1995) 117:9105-9106)

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin-producing PKS, (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3-α-mycarosyl-5-oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy-6,6α-epoxy-5-oxoerythronolide B (Donadio, S. et al. Science, (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into Saccharopolyspora erythraea, led to the production of Δ6,7-anhydroerythromycin C (Donadio S. et al. Proc. Natl. Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However, many such attempts are reported to have been unproductive (Hutchinson C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p.231), and no further examples of altered polyketides have been reported. The complete DNA sequence of the genes from

Streptomyces hygroscopicus that encode the modular Type 1 PKS governing the biosynthesis of the macrocyclic immunosuppressant polyketide rapamycin has been disclosed (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843)(Figure 3). The DNA sequence is deposited in the EMBL/Genbank Database under the accession number X86780.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used as appropriate in successive cycles (Bibb, M. J., et al. EMBO J. (1989) 8:2727-2736; Sherman, D. H. et al. EMBO J. (1989) 8:2717-2725; Fernandez-Moreno, M. A. et al. J.Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II PKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained by the introduction of cloned Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from Streptomyces coelicolor, into an anthraquinone polyketide-producing strain of Streptomyces galileus (Bartel, P. L. et al. J. Bacteriol. (1990) 172:4816-4826).

International Patent Application Number WO 95/08548 describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS clusters, to obtain hybrid polyketides. The same International Patent Application WO 95/08548 describes the construction of a strain of Streptomyces coelicolor which substantially lacks the native gene cluster for actinorhodin, and the use in that strain of a plasmid vector pRM5 derived from the low-copy number plasmid vector SCP2* isolated from Streptomyces coelicolor (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and in which heterologous PKS-containing DNA may be expressed under the control of the divergent act I/act III promoter region of the actinorhodin gene cluster (Fernandez-Moreno, M. A. et al. J.Biol. Chem. (1992) 267:19278-19290) The plasmid pRM5 also contains DNA from the actinorhodin biosynthetic gene cluster encoding the gene for a specific activator protein, Act II-orf4. The Act II-orf4 protein is required for transcription of the genes placed under the control of the act I/act III bidirectional promoter and activates expression during the transition from growth to stationary phase in the vegetative mycelium (Hallam, S. E. et. al. Gene (1988) 74:305-320).

Type II PKS clusters in Streptomyces are known to be activated by pathway-specific activator genes (Narva, K.E. and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333; Stutzman-Engwall, K. J. et al. J. Bacteriol (1992) 174:144-154; Fernandez-Moreno, M. et al. Cell (1991)

66:769-780; Takano, E. et al. Mol. Microbiol. (1992) 7:837-845; Takano, E. et al. Mol. Microbiol. (1992) 6:2797-2804) whose gene product is required for transcription from specific promoters. The gene product of the activator genes is speculated to act by binding to specific DNA sequences in promoters of the PKS gene cluster in which the activator gene is located (Stutzman-Engwall, K. J. et al. J. Bacteriol (1992) 174:144-154; Takano, E. et al. Mol. Microbiol. (1992) 7:837-845). The DnrI gene product complements a mutation in the actII-orf4 gene of S. coelicolor, implying that DnrI and ActII-orf4 proteins act on similar targets. A gene (srmR) has been described (EP 0 524 832 A2) that is located near the Type I PKS gene cluster for the macrolide polyketide spiramycin, this gene specifically activates the production of the macrolide polyketide spiramycin, but no other examples have been found of such a gene. Also, no homologues of the ActII-orf4/DnrI/RedD family of activators have been described that act on Type I PKS genes.

Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or possess completely novel bioactivity. The complex polyketides produced by modular Type I PKSs are particularly valuable, in that they include compounds with known utility as antihelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents.

Because of their structural complexity, such novel polyketides are not readily obtainable by total chemical synthesis, or by chemical modifications of known polyketides.

There is a need to develop reliable and specific ways of deploying individual modules in practice so that all, or a large fraction, of hybrid PKS genes that are constructed, are viable and produce the desired polyketide product. This is particularly true if it is desired to create large numbers of individual PKS gene sets using Type I modular PKS genes in a combinatorial fashion, where it will not be feasible to analyse all members of the set. Such libraries of polyketides offer a highly attractive alternative to the random screening of soil samples for the discovery of novel polyketides with valuable bioactive properties.

Similarly, although specific host-vector combinations have been reported that allow the controlled expression of heterologous genes in certain Streptomyces as for example using induction by added thiostrepton as described for Streptomyces lividans 66 and Streptomyces coelicolor (Takano, E. et al. Gene (1995) 166:133-137) and by utilising nutritional signals at the onset of differentiation, as for Streptomyces coelicolor in International Patent Application number WO 95/08548, there remains an important need for the development of general methods of controlling and even enhancing the expression of a structural gene, or of a set of structural genes,

that governs the biosynthesis of a potentially valuable secondary metabolite such as one of the complex polyketides, in an engineered strain of Streptomyces or of a related filamentous bacterium.

One aspect of the invention arises from our appreciation that a PKS gene assembly (particularly of type I) encodes a loading module which is followed by extension modules. Thus Fig. 2 shows the organisation of the DEBS genes. The first open reading frame encodes the first multi-enzyme or cassette (DEBS1) which consists of three modules: the loading module (ery-load) and two extension modules (modules 1 and 2). The loading module comprises an acyl transferase and an acyl carrier protein. This may be contrasted with Fig. 1 of WO93/13663 (referred to above). This shows ORF1 to consist of only two modules, the first of which is in fact both the loading module and the first extension module.

In one aspect the invention concerns the production of a hybrid PKS gene assembly comprising a loading module and at least one, and preferably a plurality, of extension modules by assembling together a first nucleic acid portion or portions encoding at least one domain of a first type I PKS with a second nucleic acid portion or portions encoding at least one type I PKS domain which is heterologous to said first PKS. Generally the nucleic acids are DNA. The first and second portions may each encode domain(s) of respective different PKS's.

Preferably the hybrid PKS encodes a loading module

and from 1 to 6 extension modules within any give cassette. More preferably there are at least 2 extension modules. NB: products resulting from many more than 6 modules can result from assemblies of synthases (c.f. rapamycin).

The first portion may encode a loading module, while the second portion encodes one or more extension modules. Alternatively the first portion(s) may encode all or part of a loading module, the first two extension modules, and a chain terminating enzyme (generally a thioesterase), e.g. of erythromycin PKS, and the second portion(s) correspond to one or more domains and/or modules of a different PKS.

It is particularly useful to provide a hybrid PKS gene assembly in which the loading module is heterologous to the extension modules and is such as to lead to a polyketide having an altered starter unit. NB: This is a concept quite unknown to the prior art since this does not recognise the existence of loading modules. WO93/13663 refers to altering PKS genes by inactivating a single function (i.e. a single enzyme) or affecting "an entire module" by deletion, insertion or replacement thereof. But in their terms the loading assembly is not a module.

If the loading module is one which accepts many different carboxylic acid units then the hybrid gene assembly can be used to produce many different polyketides. For example a hybrid gene assembly may employ nucleic acid encoding an <u>avr</u> loading module with

ery extender modules. A loading module may accept unnatural acid units. Alternatively or additionally we may alter the end of a gene assembly. Thus the normal chain terminating enzyme of a PKS (usually thioesterase) may be replaced by an enzyme leading to a different type of product. Thus use may be made of the enzyme from the rapamycin system that connects the polyketide chain to an aminoacid chain. This can be used to synthesise polypeptide/polyketide combinations, e.g. for producing β-lactam derivatives.

Of course one may make alterations within a product polyketide, particularly by replacing an extension module by one that gives a ketide unit at a different oxidation state and/or with a different stereochemistry. NB: It has generally been assumed that the stereochemistry of the methyl groups in the polyketide chain is determined by the acyltransferase. But it is in fact a feature of other domains of the PKS, and thus open to variation only by replacement of those domains, individually or by module replacement. Methyl and other substituents can be added or removed by acyltranferase domain replacement or total module replacement.

This aspect of the invention is largely concerned with treating PKS gene modules as building blocks that can be used to construct enzyme systems, and thus polyketide products, of desired types. This generally involves the cutting out and the assembly of modules and multi-module groupings. It might be assumed that the correct places

for making and breaking intermodular connections would be in the linking regions between modules. However we have found that it is preferable to make cuts and joins actually within domains (i.e. the enzyme-coding portions), close to the edges thereof. The DNA is highly conserved here between all modular PKS's, and this appears to aid in the construction of hybrids that can be transcribed. It also assists in maintaining the spacing of the active sites of the encoded enzymes, which seems to be important. For example in producing a hybrid gene by replacing the ery loading module by an avr loading module, we removed the ery module together with a small amount of the following ketosynthase (KS) domain. The start of the KS domain (well spaced from the active site) is highly conserved and therefore provides a good splicing site. The excised ery module was then replaced by an avr loading module.

In fact when substituting a loading module, it may be desirable to replace not just the loading module domains (generally acyl transferase (AT) and acyl carrier protein (ACP)) but also the KS at the start of the following extension module. Typically the excised loading module would have provided a propionate starter, and the replacement is intended to provide one or more different starters. But propionate may feed in to the KS of the extension module from a propionate pool in the host cell, leading to dilution of the desired products. This can be largely prevented by substituting an extended loading

module including all or most of the KS domain. (The splice site may be in the end region of the KS gene, or early in the following AT gene.)

Another technique which we may use in generating novel polyketides (optionally together with one or more other techniques described herein) involves the deletion or inactivation of a gene involved in the production of a material which would otherwise be incorporated into a polyketide. This can facilitate the incorporation of a different material. For example, rapamycin contains a pipecolate unit. The natural gene cluster for rapamycin biosynthesis in S.hygroscopicus includes an enzyme for producing pipecolate from a precursor, L-lysine. We have produced a mutant organism which lacks this synthetic activity. It is thus unable to produce rapamycin under normal conditions, but can do so if fed with L-pipecolic acid. More usefully it can product rapamycin analogues if fed with analogues of pipecolic acid.

After the formation of a macrolactone there are typically several late steps including oxidations, methyltransfers. By suitable manipulation of the fermentation medium, or addition of specific methylation inhibitors, or specific cytochrome P450 hydroxylase inhibitors such as metapyrone, partially processed rapamycin precursors are obtained was well. Such derivatives can be further elaborated by bioconversion using various strains of Streptomyces.

When replacing "modules", we are not restricted to

"natural" modules. For example a "combinatorial module" to be excised and/or replaced and/or inserted may extend from the corresponding domain of two natural-type modules, e.g. from the AT of one module to the AT of the next, or from KS to KS. The splice sites will be in corresponding conserved marginal regions. A combinatorial module can also be a 'double' or larger multiple, for adding 2 or more modules at a time.

The invention further provides such gene assemblies, vectors containing such gene assemblies, and transformant organisms that can express them. Transformant organisms may harbour recombinant plasmids, or the plasmids may integrate. A plasmid with an *int* sequence will integrate into a specific attachment site (*att*) of a host's chromosome. Transformant organisms may be capable of modifying the initial products, e.g. by carrying out all or some of the biosynthetic modifications normal in the production of erythromycins (as shown in Fig 2B) and/or other polyketides. Use may be made of mutant organisms such that some of the normal pathways are blocked, e.g. to produce products without one or more "natural" hydroxy-groups or sugar groups. The invention further provides novel polyketides as producible, directly or indirectly, by transformant organisms. (This includes polyketides

which have undergone enzymic modification in the organisms and/or have been isolated and subjected to chemical modification.)

In a second aspect the invention provides a hybrid gene assembly comprising structural gene components operably linked to a promoter which is not naturally linked thereto and is of a type II PKS, preferably linked to its specific cognate activator gene. Particularly preferred is the use of the act I promoter and the Act II-orf4 activator gene from *S.coelicolor*, for expression in hosts other than *S.coelicolor* (usually other actinomycetes, particularly other streptomycetes). The structural gene components may be of a type I PKS gene system.

The invention in its second aspect further provides vectors containing such gene assemblies, and transformant organisms that can express them. It is possible to combine the two aspects of the invention, so that a hybrid type I gene is expressed under the control of a type II promoter.

In a further aspect the invention provides novel polyketides obtainable by means of the previous aspects. These include the following.

(i) An erythromycin analogue (being a macrolide compound with a 14-membered ring) in which C-13 bears a side-chain other than ethyl, generally an α-branched C_2-C_5 alkyl group, a C_3-C_8 cycloalkyl or cycloalkenyl group (optionally substituted e.g. with one or more hydroxy, C_{1-4}

alkyl or alkoxy groups or halogen atoms), or a 3-6 membered heterocycle containing O or S, saturated or fully or partially unsaturated, optionally substituted (as for cycloalkyl). Preferred candidates for the C-13 substituent R are the groups of carboxylate units R. C0.0-usable as substrates by an avr starter module, or rapamycin starter variants. Preferred substrates include isobutyrate (R=i-Pr) and 2-methylbutyrate (R=1-methylpropyl). Other possibilities include n-butyrate, cyclohexyl carboxylate, cycloheptanyl carboxylate, cyclohexenyl carboxylates, cycloheptenyl carboxylates, and ring-methylated variants of the cyclic carboxylates.

The erythromycin analogue may correspond to the initial product of a PKS (6-deoxyerythronolide) or the product after one or more of the normal biosynthetic steps. As shown in Fig. 2B these comprise: 6-hydroxylation; 3-0-glycosylation; 5-0-glycosylation; 12-hydroxylation; and specific sugar methylation.

Thus the analogues include ones corresponding to 6-deoxyerythronolide B, erythromycin A, and the various intermediates and alternatives shown in Fig. 2B.

Additionally or alternatively, there may be chemical modification. For example one or more hydroxy groups may be oxidised (e.g. to produce 3-keto derivatives) or eliminated (e.g. to produce 10-ene derivatives). Some examples of chemical modifications applicable to the present inventions are those that give rise to azithromycin, roxithromycin, clarithromycin and those

disclosed in some French patents of Roussel Uclaf:2697523, 2697524 and 2702480.

(ii) erythromycin analogues differing from the corresponding 'natural' compound (Figure 2b) in the oxidation state of one or more of the ketide units (i.e. selection of alternatives from the group: -CO-, -CH(OH)-, =CH-, and $-CH_2-$).

The stereochemistry of any -CH(OH)- is also independently selectable.

(iii) erythromycin analogues differing from the corresponding 'natural' compound in the absence of a 'natural' methyl side-chain. (This is achievable by use of a variant AT). Normal extension modules use either C_2 or C_3 units to provide unmethylated and methylated ketide units. We may provide unmethylated units where methylated units are natural (and vice versa, in systems where there are naturally unmethylated units) and also provide larger units, e.g. C_4 to provide ethyl substituents.

(iv) erythromycin analogues differing from the corresponding 'natural' compound in the stereochemistry of 'natural' methyl; and/or ring substituents other than methyl.

(v) erythromycin analogues having the features of two or more of sections (i) to (iv);

(vi) triketide lactone ("TKL") analogues:

(I)

R_3 is the side-chain derived from the starter unit, and is subject to the variation described for the C-13 sidechain described above in (i).

R_1 and R_2 are "naturally" methyl but either or both may be replaced by hydrogen or ethyl (using extender units employing butyrate)

The natural stereochemistry is

(II)

but any one or two or all of R_1, R_2, R_3 and OH may have the opposite stereochemistry. Generally TKL analogues can have variations as described for erythromycins in (i) to (v) above.

(vi) polyketides of types other than erythromyin, e.g. rapamycin or avermectin, having modifications corresponding to those described in sections (i) to (v).

For example, we have produced rapamycin variants using as starters:

viii) truncated or extended versions of polyketide chains:

a) diketides R^1-CHOH-CHR^2-CO_2H

b) triketides R^1-CHOH-CHR^2-CHOH-CHR^3-CO_2H

c) tetraketides R^1-CHOH-CHR^2-CHOH-CHR^3-CHOH-CHR^4-CO_2H

d) penta-, hexa-, hepta- and larger ketide chains

The chains may have variants as described in (i) to (iv).

ix) ketide/non-ketide fusions.

Rapamycin is a natural example of a polyketide/peptide fusion. Means such as a peptide incorporating enzyme may be employed to create polyketides fused to one or more amino acids.

x) Polyketides (or fusions) cyclised by formation of lactones, hemiketals, ketals, lactams, or lactols.

xi) derivatives of any of the above which have undergone further processing by non-PKS enzymes, eg one or more of hydroxylation, epoxidation, glycosylation, and methylation.

The present invention provides a method of obtaining novel complex polyketides; and novel methods of increasing production of both new and known polyketides.

Thus in one type of embodiment of the invention, one or more segments of DNA encoding individual modules or domains within a natural Type I PKS (the "donor" PKS) have been used to replace the DNA encoding, respectively, individual modules or domains of another natural Type I

PKS (the "acceptor" PKS). The total number of extension modules assembled in the hybrid PKS is not fixed, but the preferred number of such modules in any one multienzyme or cassette ranges between one, creating the smallest possible functional PKS, and six, which equals the largest number of consecutive modules found to be housed in a single multienzyme of a natural Type I PKS, namely the rap PKS of Streptomyces hygroscopicus.

In a particularly preferred embodiment for the purposes of defining which hybrid PKS genes will be viable and productive, the acceptor PKS DNA consists of, or comprises of, the loading module, first two extension modules and chain-terminating thioesterase of the ery PKS, or other, preferably natural, type I PKS, housed in a suitable plasmid vector. Either one or more individual domains, or one or more individual modules, are specifically replaced by DNA encoding analogous domains or modules and derived from a different natural Type I PKS (the "donor" PKS). The altered DNA sequence is introduced into a suitable microorganism and the genetically engineered microorganism is cultured under conditions suitable for polyketide production.

Surprisingly and unexpectedly, these genetically engineered microorganisms when cultured under suitable conditions have been found to produce non-natural analogues of the polyketide product(s) of the natural acceptor PKS, and where appropriate the products are found to undergo the same processing as the natural polyketide.

In this aspect of the invention, the plasmid vector may be any one drawn from a long list of plasmid vectors well known to be useful for cloning in Streptomyces and related Gram positive bacteria. It has been found particularly useful to select a low copy number plasmid vector with a broad host range based on the SCP2* plasmid of Streptomyces coelicolor M110. The construction is described herein of two SCP2*-derived plasmids particularly suitable for this purpose. The triketide lactone synthase of the "acceptor" PKS may be composed of loading modules, extension modules and chain-terminating activities drawn from any natural or non-natural Type I PKS, but particularly suitable for this purpose are the components of Type I PKSs for the biosynthesis of erythromycin, rapamycin, avermectin, tetronasin, oleandomycin, monensin, amphotericin and rifamycin, for all of which the gene and modular organisation is known through gene sequence analysis, at least in part. Particularly favourable examples of the loading modules of the donor PKS are those loading modules showing a relaxed specificity, for example the loading module of the avermectin (avr)-producing PKS of Streptomyces avermitilis; or those loading modules possessing an unusual specificity, for example the loading modules of the rapamycin-, FK506- and ascomycin-producing PKSs, all of which naturally accept a shikimate-derived starter unit.

Genetically-engineered cells suitable for expression

of hybrid Type I PKS genes may be drawn from any actinomycete capable of maintaining the vector in either autonomous or integrated form. Particularly effective hosts are Saccharopolyspora erythraea, Streptomyces coelicolor, Streptomyces avermitilis, Streptomyces griseofuscus, Streptomyces cinnamonensis, Micromonospora griseorubida, Streptomyces hygroscopicus, Streptomyces fradiae, Streptomyces longisporoflavus, Streptomyces lasaliensis, Streptomyces tsukubaensis, Streptomyces griseus, Streptomyces venezuelae, Streptomyces antibioticus, Streptomyces lividans, Streptomyces rimosus and Streptomyces albus. These include hosts in which SCP2*-derived plasmid vectors are known to replicate autonomously, as for example S. coelicolor, S. avermitilis and S. griseofuscus; and other hosts such as Saccharopolyspora erythraea in which SCP2*-derived plasmids become integrated into the chromosome through homologous recombination between sequences on the plasmid insert and on the chromosome.

In a further aspect of the present invention, a plasmid containing "donor" PKS DNA is introduced into a host cell under conditions where the plasmid becomes integrated into an acceptor PKS genes on the bacterial chromosome by homologous recombination, to create a hybrid PKS. A preferred embodiment is when the donor PKS DNA includes a segment encoding a loading module, in such a way that this loading module becomes linked to the acceptor PKS genes on the chromosome. Such a hybrid PKS

produces valuable and novel hybrid polyketide products when cultured under suitable conditions as described herein. Specifically, when the loading module of the acceptor PKS is replaced by the loading module of the avermectin-producing (avr) PKS, the hybrid polyketide products contain a starter unit typical of those used by the avr PKS. Thus when the loading module of the ery PKS is replaced by the avr loading module, Saccharopolyspora erythraea strains containing such hybrid PKS are found to produce 14-membered macrolides containing starter units typically used by the avr PKS.

It is very surprising and unexpected that the 14-membered macrolide polyketides produced by such recombinant cells of S. erythraea are found to include derivatives of erythromycin A, showing that the several processing steps required for the transformation of the products of the hybrid PKS into novel and therapeutically valuable erythromycin A derivatives are correctly carried out.

A further aspect of the present invention is the unexpected and surprising finding that transcription of any of the hybrid Type I PKS genes, whose construction is described herein, can be specifically increased when the hybrid genes are placed under the control of a promoter for a Type II PKS gene linked to a specific activator gene for that promoter. It is particularly remarkable that when a genetically engineered cell containing hybrid Type I genes under such control is cultured under conditions

suitable for polyketide production, significantly enhanced levels of the hybrid polyketide are produced. Such specific increases in yield of a valuable polyketide product are also seen for natural polyketides produced by a Type I PKS placed under the control of a Type II PKS promoter and activator gene. In a preferred embodiment, Type I PKS genes present on an SCP2*-derived plasmid are placed under the control of the bidirectional actI promoter derived from the actinorhodin biosynthetic gene cluster of Streptomyces coelicolor, and in which the vector also contains the structural gene encoding the specific activator protein Act II-orf 4. The recombinant plasmid is introduced into bacterial hosts other than Streptomyces coelicolor chosen from Streptomyces and related genera, under conditions where either the introduced PKS genes, or PKS genes already present in the host strain, are expressed under the control of the actI promoter.

The recombinant strains produce the desired specific polyketide product and the activator gene requires only the presence of the specific promoter in order to enhance transcriptional efficiency from the promoter. This is particularly surprising in that activators of the ActII-orf4 family do not belong to a recognised class of DNA-binding proteins. Therefore it would be expected that additional proteins or other control elements would be required for activation to occur in a heterologous host not known to produce actinorhodin or a related

isochromanequinone pigment. It is also surprising and useful that the recombinant strains produce up to 10-fold more specific polyketide product than when the same PKS genes are under the control of the natural promoter, and the specific polyketide product is also produced precociously in growing culture, rather than only during the transition from growth to stationary phase. Such polyketides are useful as antibiotics, anti-cancer agents, immunosuppressants and for many other purposes in human and veterinary medicine.

When the genetically engineered cell is Saccharopolyspora erythraea, the activator and promoter are derived from the actinorhodin PKS gene cluster and the actI/actII-orf4-regulated ery PKS gene cluster is housed in the chromosome, following the site-specific integration of a low copy number plasmid vector, culturing of these cells under suitable conditions produces up to ten fold more total 14-membered macrolide product than in a comparable strain not under such heterologous control. When in such a genetically engineered cell of S. erythraea the PKS genes under this heterologous control are hybrid Type I PKS genes whose construction is described herein, then again up to ten-fold more hybrid polyketide product is obtained compared to the same hybrid Type I PKS genes not under such control. Specifically, when the hybrid Type I PKS genes are the ery PKS genes in which the loader module is replaced by the avr loading module, a ten-fold increase is found in the total amounts of novel

14-membered macrolides produced by the genetically engineered cells when cultured under suitable conditions as described herein.

The suitable and preferred means of growing the genetically engineered cells, and the preferred means of isolating both the natural and the hybrid polyketides are described more fully in the Examples.

Some embodiments of the invention will now be described with reference to the accompanying drawings in which:

Fig. 1 gives the chemical formulae of three known polyketides;

Fig. 2A is a diagram showing the functioning of 6-deoxyerythronolide synthase B (DEBS), a PKS producing 6-deoxyerythronolide B (6-DEB), a precursor of erythromycin A;

Fig. 2B shows post-PKS biosynthesis of erythromycins including the conversion of 6-DEB to erythromycin A;

Fig. 3 is a diagram showing the biosynthesis of rapamycin;

Fig. 4 is a diagram showing the construction of plasmid pDEL702;

Fig. 5 is a diagram showing the construction of plasmid pCJR101;

Fig. 6 is a diagram showing the construction of plasmid pCJR110;

Fig. 7 is a diagram showing the construction of plasmid pNTEP2;

Fig. 8 is a diagram showing the construction of plasmid pRMTE and pCJRTE;

Fig. 9 a and b is a diagram showing the construction of plasmid pIG1;

Fig. 10 is a diagram showing the construction of plasmid pKW15; this includes DNA encoding a loading module, a first extender module, and the chain-terminating thioesterase, capable of receiving modules;

Fig. 11 is a diagram showing the construction of plasmid pAR33;

Fig. 12 is a diagram showing the construction of plasmid pAR8;

Fig. 13 is a diagram showing the construction of plasmid pE1A2TE;

Fig. 14 is a diagram showing the construction of plasmid pMO7;

Fig. 15 is a diagram showing the construction of plasmid pAVLD;

Fig. 16 shows the integration of pAVLD into the genome of *S.erythraea* NRRL2338; and

Fig. 17 shows the integration of pAVLD into the genome of *S.erythraea* TER43.

The present invention will now be illustrated, but is not intended to be limited, by means of some examples.

Use was made of the following media and solutions.

Sucrose-Succinate defined medium

sucrose	69g
KNO_3	10g

succinic acid	2.36g
KH_2PO_4	2.7g
$MgSO_4.7H_2O$	1.2g
$ZnCl_2$	10mg
$MnCl_2.4H_2O$	6.2g
$CuCl_2.2H_2O$	0.53mg
$CoCl_2$	0.55mg
$FeSO_4.7H_2O$	2.5mg
$CaCl_2.2H_2O$	38mg
milli-Q water	to 1l
KOH	To pH 6-6.4

YEME

sucrose	340g
yeast extract	3g
peptone	5g
malt extract	3g
glucose	10g
water	to 1.0l

Tap water medium

glucose	5g
tryptone	5g
yeast extract	2.5g
EDTA	36mg
tap water	to 1.0 l
KOH	to pH7.1

after sterilisation: 2.5M $MgCl_2$ 2ml

Trace elements solution: $ZnCl_2$, 40mg/l; $FeCl_3.6H_2O$, 200mg/l; $CuCl_2.2H_2O$, 10 mg/l; $MnCl_2.4H_2O$, 10mg/l; $Na_2B_4O_7.10H_2O$), 10mg/l; $(NH_4)_6MO_7O_{24}.4H_2O$, 10mg/l; 4mg/ml lysozyme.

BW1 medium

$CaCO_3$	2 g
Difco tryptone	2.5 g
soy flour	5 g
Difco yeast extract	5 g

soluble starch (Sigma) 20 g

pH 7.2

K_2HPO_4	1.2	g
$MgSO_4.7H_2O$	1.2	g
$FeSO_4.7HO_2O$	0.012	g
$MnSO_4$	0.0012	g
$ZnSO_4.7H_2O$	0.0012	g
Tap water	to 1.0	l

BW2 Medium

$CaCO_3$	7	g
soy flour	5	g
Difco yeast extract	5	g
soluble starch (Sigma)	80	g
K_2HPO_4	1	g
$MgSO_4.7H_2O$	1	g

10 ml of a trace elements solution as for tap water medium.

Made up to 1 litre with distilled water. pH adjusted to 7.2.

Example 1

Construction of strain Saccharopolyspora erythraea JC2

An S. erythraea host cell, genetically engineered to remove all of the native eryA genes which encode the

erythromycin-producing type I PKS, except for the region of eryAIII DNA encoding the chain-terminating thioesterase, was constructed by homologous recombination starting from S. erythraea NRRL2338. S. erythraea NRRL2338 is a wild-type erythromycin-producing strain obtained from the Northern Regional Research Laboratories, Peoria, Illinois, USA, under the above designation. The ery cluster is made up of the PKS genes, flanked by other genes involved in later stages of erythromycin biosynthesis, including those involved in glycosylation, hydroxylation and methylation.

Plasmid pDEL was constructed as follows (Figure 4). The 1.4 kbp SmaI segment containing the start codon of eryAI was cloned into pUC18 to give p612SL, the segment was excised as a BamHI-SacI fragment using the multiple cloning sites of pUC18, and subcloned into a derivative of plasmid pT7-18 (Roberts, G. A. et al. Eur. J. Biochem. (1993) 214:305-311)) containing the SacI/KpnI fragment of eryAIII that encodes the C-terminus of DEBS3 from which a BglII-SacI fragment had been excised. The identity of plasmid pDEL was confirmed by restriction analysis.

Plasmid pDEL was digested with BglII and treated with calf intestinal alkaline phosphatase, and ligated to plasmid pIJ702 (Katz, E. et al. J. Gen. Microbiol. (1983) 129:2703-2714) which had been linearised with BglII. The resulting mixture contains the desired plasmid pDEL702.

Protoplasts of S. erythraea NRRL2338 (Yamamoto, H. et al. J. Antibiot. (1986) 39:1304-1313) were transformed

with 10 μg pDEL702 and stable thiostrepton resistant colonies were isolated. Individual colonies were selected and subcultured four times in non-selective liquid medium (tryptone soy broth) followed by preparation and regeneration of protoplasts. Thiostrepton sensitive colonies were isolated and characterised by restriction analysis and Southern hybridisation. One such colony was designated JC2. S. erythraea strain JC2 has been deposited at the National Collection of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen, Scotland AB2 1RY, under the designation NCIMB 40802.

Example 2

Construction of the Recombinant Vector pCJR101

pCJR101 (Figure 5) is a shuttle plasmid constructed to be used for expression of PKS genes in actinomycetes. It includes a ColEI replicon to allow it to replicate in E. coli, an SCP2* low copy number Streptomyces replicon (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and the actII-orf4 activator gene from the act cluster which activates transcription from the act promoter during the transition from growth phase to stationary phase in the vegetative mycelium. It is constructed as follows: an approximately 970 bp DNA fragment from pMF1015 (containing the actII-orf4 activator gene) (Fernandez-Moreno, M. A. et al. Cell (1991)

66:769-780) is amplified by PCR, using as primers the synthetic oligonucleotides:
5'-ACT AGT CCA CTG CCT CTC GGT AAA ATC CAG C-3' and 5'-CTT AAG AGG GGC TCC ACC GCG TTC ACG GAC-3', which also introduces flanking SpeI and AflII restriction sites. This fragment is end-repaired and introduced by blunt-ended ligation into the AatII site of plasmid pUC19 to yield plasmid p18.14. An approximately 215 bp DNA fragment is amplified from pMV400 which contains the bidirectional promoter pair PactIII/PactI) (Parro, V. et al. Nucl. Acids Res. (1991) 19:2623-2627), using as primers the synthetic oligonucleotides 5'-ACA TTC TCT ACG CCT AAG TGT TCC CCT CCC TGC CTC-3' and 5'-GTG ATG TAT GCT CAT ATG TGT CCT CCT TAA TTA ATC GAT GCG TTC GTC CGG TG-3', which also introduces flanking NdeI and AflII sites. The PCR product is digested with NdeI and AflII and ligated with the plasmid p18.14 previously cut with NdeI and AflII, to generate plasmid p19.4. A 1.1 kbp HindIII-SphI fragment containing the tsr gene, which confers resistance to thiostrepton, is obtained by PCR from plasmid pIJ922 (Lydiate, D. J. et al. Gene (1985) 35:223-235) as template, using as primers the oligonucleotides 5'-TGA ACA CCA AGC TTG CCA GAG AGC GAC GAC TTC CCC-3' and 5'-GAC AGA TTG CAT GCC CTT CGA GGA GTG CCC GCC CGG-3' which also introduces flanking HindIII and SphI sites. The PCR product is digested with HindIII and SphI and ligated with plasmid p19.4 cut with HindIII and SphI to obtain plasmid p20.5. The plasmid pIJ922 is digested with BamHI and SstI

and the fragment containing a portion of the fertility locus and the origin of replication (Lydiate, D. J. et al. Gene (1985) 35:223-235) is ligated into pUC19 digested with BamHI and Sst I to generate the bifunctional plasmid 16/2.2 (14.7 kbp). Plasmid 20.5 is digested with SalI and SphI, the two larger fragments from the digest are purified by gel electrophoresis, and combined in a four-component ligation with plasmid 16/2.2 which has been digested with XhoI and SphI. The ligation mixture is used to transform Streptomyces lividans and colonies are selected in the presence of thiostrepton. One such colony is shown to contain the desired plasmid pCJR101 (approx. 12.4 kbp), identified by its restriction pattern.

Example 3

Construction of plasmid pCJR110

The construction of plasmid pCJR110 is illustrated in Figure 6. A 1.1 kbp HindIII-XhoI fragment containing the tsr gene, which confers resistance to thiostrepton, is obtained by PCR from plasmid pIJ922 as template, using as primers the oligonucleotides 5'-TGA ACA CCA AGC TTG CCA GAG AGC GAC GAC TTC CCC-3' and 5'-GAC AGA TTC TCG AGC CTT CGA GGA GTG CCC GCC CGG-3' which also introduces flanking HindIII and XhoI sites. The PCR product is digested with HindIII and XhoI and ligated with plasmid 16/2.2 which has been digested with HindIII and XhoI, to generate plasmid

22.1. Plasmid 22.1 is digested with HindIII and SphI and ligated with plasmid 19.4 which has been digested with HindIII and SphI, to produce the desired plasmid pCJR110 (approx. 12.4 kbp), identified by its restriction pattern. Plasmid pCJR110 differs from pCJR101 in the orientation of the tsr gene, the actII-orf4 gene and the actI/actIII promoter, with respect to the SCP2*-derived origin of replication.

Example 4

Construction of plasmid pRM52

Plasmid pRM52 is a derivative of plasmid pRM5 (McDaniel, R. et al. Science, (1993) 262:1546-1550). pRM5 was first linearised by digestion with NdeI, end-repaired and then religated to produce pRM51. pRM51 was cut with PacI and NsiI and the large PacI-NsiI fragment was isolated and ligated to a short double-stranded oligonucleotide linker containing an NdeI site and constructed from the synthetic oligonucleotides 5'-TAAGGAGGACACATATGCA-3' and 5'-TAATTCCTCCTGTGTAT-3' which were annealed together. The ligation mixture was transformed into E. coli TGIrecO and isolated colonies were screened for their plasmid content. The desired plasmid (19.6 kbp) was identified by its restriction map and was designated pRM52.

Example 5

Construction of plasmid pNTEP2

Plasmid pNTEP2 contains the entire open reading frame for the chimaeric DEBS1 plus thioesterase gene, with a unique NdeI site at the start codon and unique XbaI and HindIII sites immediately 3' of the stop codon. It is constructed via several intermediate plasmids as follows (Figure 7):

Construction of plasmid pTENCO11

A library of total DNA from S. erythraea TED8 (Cortes, J. et al. Science (1995) 268: 1487-1489) was constructed in the vector λDASH II (Stratagene) and probed with eryA gene fragments. One recombinant bacteriophage designated λ-4B had an insert extending from 700 bp upstream of the eryAI start codon to the thiostrepton resistance gene of the integrated plasmid in S. erythraea TED8.

The λ-4b DNA was digested with NcoI and the 12 kbp NcoI fragment was end-repaired and ligated into SmaI-cut pUC18 and transformed into E. coli TG1recO. Individual colonies were screened for their plasmid content and one plasmid bearing the NcoI insert was selected and designated pTENCO11.

Construction of plasmid pNK8

A 4.0 kb KpnI fragment extending from 1.4 kbp upstream of the correct eryAI start codon as previously determined (Caffrey, P. et al. FEBS Letters (1992) 304:225-228), to

2.6 kbp inside the eryAI gene of S. erythraea, was excised from plasmid pBK25 (Bevitt, D. J. et al. Eur. J. Biochem. (1992) 204:39-49) and cloned into pTZ18R to obtain plasmid pBK6.12. DNA of this plasmid was used as the template for a PCR reaction to obtain a 360 bp product in which a unique Nde I site is created at the start codon of eryAI and a unique SmaI site is created at the other end of the PCR product. The oligonucleotides used were 5'-CCC ATA TGG CGG ACC TGT CAA AGC-3' and 5'-ATT GCG CGC CCT GGC CCG GGA A-3'. The product was end-repaired and ligated into SmaI cut pUC18, and transformed into E. coli TG1recO.

Individual colonies were screened for their plasmid content and one plasmid bearing the insert in an orientation such that the SmaI site was adjacent to the KpnI site of the polylinker was selected and designated plasmid pNDE6. Plasmid pNDE6 was digested wth SmaI and KpnI, and ligated with a 2.3 kbp fragment of the eryAI gene obtained by digestion of plasmid pBK6.12 with SmaI and KpnI. The ligation mixture was used to transform E. coli TG1recO and individual colonies were screened for their plasmid content. A plasmid containing the desired 2.6 kbp NdeI-KpnI fragment was isolated and designated plasmid pNDE7. The NdeI-KpnI insert was excised from plasmid pNDE7 and ligated into plasmid pT7-18, previously digested with NdeI and KpnI. Plasmid pT7-18 is a derivative of plasmid pT7-7 (Tabor, S. and Richardson, C.C. Proc. Natl. Acad. Sci. USA (1985) 82:1074-1078) in

which the polylinker is replaced by the polylinker from pUC18. The ligation mixture was used to transform E. coli TG1recO and individual colonies were screened for their plasmid content and one plasmid containing the desired 2.6 kbp NdeI-KpnI insert was selected and designated pNK8.

Construction of plasmid pNTE5

Plasmid pNK8 was transformed into a methylation-deficient strain of E. coli ET12567 (MacNeil, D.J. et al. Gene (1992) 111:61-68) and the plasmid pNK8 was isolated from this strain and digested with ClaI . An 11 kbp ClaI fragment obtained by digestion of pTENC011 was ligated into the digested pNK8 and transformed into E. coli TG1recO. Individual colonies were screened for their plasmid content and one plasmid, in which the 11 kbp insert was correctly oriented to regenerate the reading frame of eryAI, was selected and designated pNTE5.

Construction of plasmid pNTEP2

A ClaI-EcoRI polylinker, bearing unique restriction sites for XbaI and for HindIII was constructed, from the following complementary synthetic oligonucleotides:

5'-AATTCATAGTCTAGAAGCTTAT-3'

and

5'-CGATAAGCTTCTAGACTATG-3'

The polylinker was ligated into plasmid pNTE5, which had been digested with ClaI and EcoRI to remove a 2.3 kbp ClaI-EcoRI fragment. The ligation mixture was used to transform E. coli TG1recO and individual colonies were screened for their plasmid content. One plasmid

containing the polylinker was identified and designated pNTEP2.

Example 6

Construction of plasmid pRMTE

Plasmid pNTEP2 (14 kbp) was digested with NdeI and XbaI and the insert was purified by sedimentation on a sucrose gradient. The purified insert was ligated into plasmid pRM52 (19.6 kbp) (Example 4) which had been digested with NdeI and XbaI, and the vector purified by sedimentation on a sucrose gradient. The ligation mixture was used to transform E. coli and individual colonies were checked for their plasmid content. The desired plasmid pRMTE (31.5 kbp) was identified by its restriction pattern (Figure 8).

Example 7

Construction of plasmid pCJRTE

Plasmid pNTEP2 (Example 5) is digested with NdeI and XbaI and the insert is purified by sedimentation on a sucrose gradient. The purified insert is ligated into plasmid pCJR101 (12.4 kbp) which has been digested with NdeI and XbaI, and purified by sedimentation on a sucrose gradient. The ligation mixture is used to transform E. coli DHB10 and individual colonies are screened for their plasmid

content. The desired plasmid pCJRTE (24.3 kbp) is identified by its restriction pattern (Figure 8).

Example 8

Construction of S. avermitilis ATCC 31272/pCJRTE and production of triketide lactone ("TKL") derivatives therewith.

(i) Construction

Approximately 5 μg of plasmid pCJRTE is transformed into protoplasts of S. avermitilis ATCC 31272 and stable thiostrepton resistant colonies are isolated. Several such colonies are analysed for their content of plasmid DNA. A colony containing a plasmid whose restriction map shows it to be identical to pCJRTE, is designated S. avermitilis ATCC 31272/pCJRTE.

(ii) Production of (Ac)-TKL and TKL

S. avermitilis ATCC 31272/pCJRTE is inoculated into medium BW1 containing 50 μg/ml thiostrepton, and allowed to grow for four days at 28-30°C. After this time, 15 ml of the cell suspension is used to inoculate 150 ml of liquid medium BW2 containing 50μg/l thiostrepton, and allowed to grow for 6 days. After this time the cells are removed by centrifugation, washed with water, and the supernatants are combined and extracted three times with ethyl acetate (250 ml). The combined ethyl acetate extracts are washed with an equal volume of saturated sodium chloride, dried

over anhydrous sodium sulphate and the ethyl acetate is removed by evaporation under reduced pressure. Samples of the residue are taken up in a minimal quantity of diethyl ether, filtered through a plug of silica, and analyzed by GC, which reveals the presence of both (Ac)-TKL and TKL (Formula II: R_1=R_2=Me; R_3=Me for (Ac)-TKL, and Et for TKL), with identical retention times to authentic synthetic samples.

Example 9

Construction of plasmids pIG1 and pIG101

Plasmids pIG1 and pIG101 each consist of an SCP2*-derived plasmid containing a hybrid Type I PKS gene comprising the avr loading module in place of the ery loading module, the first two extension modules of the ery PKS and the thioesterase of the ery PKS. These are constructed via several intermediate plasmids as follows (Figure 9).

Construction of plasmid pVE 3.4

Plasmid pVE1446 which contains a portion of the avermectin (avr) PKS genes was obtained from E. coli strain ATCC 68250 (MacNeil, D. J. et al. Ann. N. Y. Acad. Sci. (1994) 721:123-132). Plasmid pVE1446 was digested with BamHI and the 7.6 kbp fragment between coordinates 32.15 and 3.40 (MacNeil, D. J. et al. Ann. N. Y. Acad. Sci. (1994) 721:123-132) was purified by gel electrophoresis and

recircularised. The mixture contained the desired plasmid pVE3.4 which was isolated after transformation of E. coli strain TG1recO.

Construction of plasmid pNCO12

Plasmid pBK25 (Bevitt, D. J. et al. Eur. J. Biochem. (1992) 204:39-49) was digested with NcoI and the 12 kbp fragment was end-repaired and ligated into plasmid pUC18 which had been linearised with SmaI. The ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pNCO12 was identified by its restriction pattern.

Construction of plasmid pCRabc

Plasmid pCRabc (Figure 9) was constructed as follows. Three separate PCR reactions were conducted: First, 20 pmol each of synthetic oligonucleotides A1 (5'-CTC GTC GGT GGC TTT GCG-3') and A2 (5'-CCC GGG AAA AAC GAA GAC TAG TGG CGC GGA CGG CCG-3') were used to amplify a 1.0 kbp product from 100 ng pNCO12 template. The PCR product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRa. Secondly, 20 pmol each of synthetic oligonucleotides C1 (5'-CAC GCG CAG CGC GGC GGA-3') and C2 (5'-CGAA CCG CTA GCG GTC GTC GCG ATG GCC T-3') were used to amplify a 1.5 kbp product from 100 ng

pNC012 template. The product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRc. Thirdly, 20 pmol each of synthetic oligonucleotides B1 (5'-GTGGCCCGGCCGTCCGCGCCACTAGTCTTCGTTTTT-3') and B2 (5'-AAC AGCTAGCGGTTCGTCCGCCGCTGCCGTGCC-3') were used to amplify a 1.4 kbp product from 100 ng pVE3.4 template. The product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRb.

Plasmid pCRa was digested with HindIII and SpeI and the 1.0 kbp insert was ligated with plasmid pCRb previously digested with HindIII and SpeI, to obtain plasmid pCRab. Plasmid pCRc was digested with NheI and EcoR1 and the 1.5 kbp insert was ligated with plasmid pCRab previously digested with NheI and EcoR1 to obtain plasmid pCRabc.

Construction of plasmid pNEWAVETE

Plasmid pCRabc was digested with MfeI and SfiI and the DNA fragment containing the loading domain of the avr PKS was purified by gel electrophoresis and ligated with plasmid pNTEP2 which had been digested with MfeI and SfiI and the larger fragment purified by gel electrophoresis. The ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pNEWAVETE (13.7 kbp) was identified by its restriction pattern.

Construction of plasmid pIG1

Plasmid pNEWAVETE was digested with NdeI and XbaI and the insert was purified by sedimentation on a sucrose gradient. The purified insert was ligated into plasmid pRM52 (19.6 kbp) which had been digested with NdeI and XbaI, and the vector purified by sedimentation on a sucrose gradient. The ligation mixture was used to transform E. coli and individual colonies were checked for their plasmid content. The desired plasmid pIG1 was identified by its restriction pattern.

Construction of plasmid pIG101

Plasmid pNEWAVETE is digested with NdeI and XbaI and the insert is purified by sedimentation on a sucrose gradient. The purified insert is ligated into plasmid pCJR101 (Example 2) which has been digested with NdeI and XbaI, and purified by gel electrophoresis. The ligation mixture is used to transform E. coli DHB10 and individual colonies are screened for their plasmid content. The desired plasmid pIG101 is identified by its restriction pattern.

Example 10

Construction of S. coelicolor CH999/pIG1 and production of TKL derivatives.

(i) Construction

Plasmid pIG1 which had been isolated from E. coli ET12567 (MacNeil. D. J. et al. Gene (1992) 111:61-68) was used to transform protoplasts of S. coelicolor CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG1 was confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using S. coelicolor CH999/pIG1

S. coelicolor CH999/pIG1 was inoculated into 100 ml YEME medium containing 50 μg/ml thiostrepton and allowed to grow for five days at 28-30°C. After this time the broth was filtered to remove mycelia. The broth was extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure. The residue was taken up in ethyl acetate and filtered through a plug of silica, the ethyl acetate was again removed and the residue was taken up in diethyl ether and subjected to flash chromatography on a column of silica gel eluted with diethyl ether. A fraction containing (s-pent)-TKL and (i-but)-TKL was separated from a fraction containing TKL, with minor amounts of (Ac)-TKL in a third fraction. The compounds were identified by their co-migration with authentic standards on GC analysis (25m column, programmed for 2

minutes at 70°C, then ramped to 250°C over 24 minutes. The retention times for (s-pent)-TKL, (i-but)-TKL, TKL and (Ac)-TKL were 14.9, 13.6, 12.9 and 11.9 minutes respectively. GC, electrospray MS and 1H-NMR were used to show that the major component (50-60%) was TKL.

Example 11

Construction of S. coelicolor CH999/pIG101 and production of TKL derivatives

(i) Construction

Plasmid pIG101 which has been isolated from E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is used to transform protoplasts of S. coelicolor CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies are checked for their plasmid content and the presence of plasmid pIG101 is confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using S. coelicolor CH999/pIG101

S. coelicolor CH999/pIG101 is inoculated into YEME medium containing 50 mg/ml thiostrepton and allowed to grow for five days at 28-30°C. The broth is extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts are dried over anhydrous sodium

sulphate, and the ethyl acetate is removed under reduced pressure. The residue was treated as in Example 10 and gave similar results.

Example 12

Construction of S. avermitilis ATCC31272/pIG1 and production of TKL derivative

(i) Construction

Plasmid pIG1 which had been isolated from E. coli ET12567 MacNeil, D. J. et al. Gene (1992) 111:61-68) was transformed into protoplasts of S. avermitilis ATCC31272 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG1 was confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using S. avermitilis ATCC31272/pIG1

S. avermitilis ATCC31272/pIG1 was first inoculated into medium BW1 containing 50 μg/ml thiostrepton, and allowed to grow for four days at 28-30°C. After this time 20 ml of the broth is used to seed 150 ml of medium BW2 containing 50μg/ml of thiostrepton.

The inoculated organism was then allowed to grow for 10-12

days. The broth was filtered to remove mycelia, and extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 10 mg crude product per litre. The residue was dissolved in ethyl acetate, passed through a plug of silica, and the solvent was removed. The residue was dissolved in diethyl ether and subjected to flash chromatography on a silica column (1 cm x 15 cm) eluted with diethyl ether, and fractions of 10 ml each were collected and assayed by GC. The diethyl ether was evaporated to leave about 10 mg of oily residue containing triketide lactones. The major component (50-60%) was (s-pent)-TKL, with (i-but)-TKL, TKL and (Ac)-TKL also present (i.e. compounds of formula II with R_1=R_2=Me, and R_3=1-methylpropyl ((s-pent)-TKL), i-Pr((i-But)-TKL), Et(TKL) and Me((Ac)-TKL).

Example 13

Construction of S. avermitilis ATCC31272/pIG101 and production of TKL derivatives

(i) Construction

Plasmid pIG101 which has been isolated from E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is transformed into protoplasts of S. avermitilis ATCC31272 and stable thiostrepton resistant colonies are isolated.

Individual colonies are checked for their plasmid content and the presence of plasmid pIG101 is confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using S. avermitilis ATCC31272/pIG101

S. avermitilis ATCC31272/pIG101 is first inoculated into medium BW1, described above and allowed to grow for 10-12 days. Isolation of products as in the previous example gives a fraction containing (s-pent)-TKL and (i-but)-TKL, a fraction containing TKL, and a third fraction with minor amounts of (Ac)-TKL. The compounds are identified by their co-migration with authentic standards on GC analysis.

Example 14

Construction of S. erythraea JC2/pIG1 and production of TKL derivatives

(i) Construction

Approximately 5 μg of plasmid pIG1 is transformed into protoplasts of S. erythraea JC2 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated

specifically into the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Production of triketide lactones using S. erythraea JC2/pIG1

S. erythraea JC2/pIG1 is inoculated into tap water medium containing 50 μg/ml thiostrepton and allowed to grow for four days at 30°C. After this 20 ml of the mycelium is used to seed 500 ml of sucrose-succinate medium containing 50 μg/ml thiostrepton, in a 2L flask with a single spring to reduce clumping, shaken at 280 rpm. After between 3.5 and 6 days, the broth is filtered to remove mycelia and then extracted three times with a quarter volume of ethyl acetate. The combined ethyl acetate extracts are dried over anhydrous sodium sulphate and solvent removed by evaporation. Analysis of the product mixture using GC and electrospray MS revealed that of a total of 5-6 mg/L of triketide lactone products, the major component was (s-pent)-TKL (about 1.5 mg/L), with other components present being (i-but)-TKL, TKL and a minor amount of (Ac)-TKL.

Example 15

Construction of S. erythraea JC2/pIG101 and production of TKL derivatives

(i) Construction

Approximately 5 μg of plasmid pIG101 is transformed into protoplasts of S. erythraea JC2 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Production of triketide lactones using S. erythraea JC2/pIG101

The same procedure as in Example 14 (ii) was followed. Analysis of the product mixture using GC and electrospray MS revealed that of a total of 5-6 mg/L of triketide lactone products, the major component was (s-pent)-TKL (about 1.5 mg/L), with other components present being (i-but)-TKL, TKL and a minor amount of (Ac)-TKL.

Example 16

Construction of plasmid pKW15

Plasmid pKW15 is a pT7-derived vector containing an insert comprising the loading module, the first extension module and the thioesterase of the ery PKS, suitable for

subcloning into an SCP2*-based vector to obtain expression of a diketide synthase gene; and also suitable for insertion of heterologous DNA containing one or more intact modules. Plasmid pKW15 is obtained via several intermediate plasmids as follows (Figure 10).

Construction of plasmid pKW11

Plasmid pNTEP2 (Example 5) was digested with BglII, the sticky ends were filled in and religated, to produce plasmid pKW11. The insert in plasmid pKW11 consists of a chimaeric eryAI-eryAIII gene encompassing the loading didomain, module 1 and module 2 from DEBS1 and the thioesterase from DEBS3. The strategy to obtain a 'diketide synthase' was to remove the DNA encoding part of module 1, the whole of module 2 and part of the thioesterase, by digestion of plasmid pKW11 with EcoRV and EcoR1, and then to reconstitute module 1 and the N-terminal part of ACP1 by insertion of an appropriate PCR product, and similarly a PCR product was designed to replace the C-terminal part of ACP2 and the thioesterase. The two PCR products are joined by a unique BglII site created in the active site of the ACP, which involves an alteration in amino acid sequence of the hybrid ACP domain from EL (glutamic acid followed by leucine) as found in both ACP1 and ACP2 domains, to DL (aspartic acid followed by leucine). Such alterations in sequence at a PKS active site, with a view to retaining function, have not been previously attempted and it is not obvious that such

altered sites should remain active.

Construction of plasmids pKW12, pKW13, pKW14 and pKW15

For the PCR amplification of DNA for module 1, the following synthetic oligonucleotides were used as mutagenic primers, one containing an EcoRV site and the other a BglII site:

5'-GCAGGGATATCGCACGTTCCTGG-3'

and 5'-CGCCGAGATCTGCGAAGGCCTGGTCGGCGGG-3'

PCR was carried out on pNTEP2 as template using Pfu DNA polymerase and 30 cycles of 95° (1 min); annealing at 55°C (1 min) and extension at 72°C (2 min), in the presence of 10% (vol/vol) dimethylsulphoxide. The product (PCR1) was end-repaired and cloned into SmaI-cut phagemid pUC119 and the ligation mixture was used to transform E. coli TG1recO. Plasmid DNA was prepared from individual colonies and the desired plasmid (5.0 kbp) was identified by its restriction pattern and was designated pKW12.

For PCR amplification of the DNA for the 5' end of module 2 and the thioesterase domain, the following oligonucleotides containing respectively a Bgl II site and an EcoRI site, were used as mutagenic primers:

5'-ATGAATTCCCTCCGCCCAGCCAG-3'

and

5'-ACAGATCTCGGCTTCGACTCGCTGACCG-3'

PCR was carried out on pNTEP2 as template exactly as described above for PCR1 and the product (PCR2) was end-repaired and cloned into SmaI-cut phagemid pUC119. The ligation mixture was used to transform E. coli TG1recO and plasmid DNA was prepared from individual colonies. The desired plasmid (4.1 kbp) was identified by its restriction pattern and was designated pKW13.

Plasmid pKW12 was digested with EcoRV and HindIII, and the 1.8 kbp insert was end-repaired, and then ligated together with plasmid pKW11 which had been linearised with EcoRV and treated with alkaline phosphatase. The ligation mixture was transformed into E. coli TG1recO and the plasmid content of individual colonies was checked. The desired plasmid (15.8 kbp) was identified in which the unique Eco RV site had been reconstituted, and this plasmid was designated pKW14.

Plasmid pKW13 was digested with BglII and EcoRI and the 0.9 kbp insert was ligated into plasmid pKW14 which had been digested with BglII and EcoRI. The ligation mixture was transformed into E. coli TG1recO and the plasmid content of the individual colonies was checked. The desired plasmid (9.32 kbp) was identified, in which the 0.9 kbp BglII-EcoRI fragment of pKW13 replaced the 9.5 kbp BglII-EcoRI segment of pKW14, and this plasmid was

designated pKW15.

Example 17

Construction and use of plasmid pKW16

(i) Construction

Plasmid pKW15 was digested with NdeI and XbaI and the insert was ligated into plasmid pRM52 which had also been digested with NdeI and XbaI. The ligation was transformed into E. coli TGI recO and isolated colonies were screened for their plasmid content. The desired plasmid was identified by its restriction map and was designated pKW16.

(ii) Use of plasmid pKW16 for construction of S. coelicolor CH999/pKW16

Plasmid pKW16 was used to transform the methylation-deficient strain E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) and the demethylated plasmid pKW16 DNA isolated from this strain was used to transform S. coelicolor CH999 (McDaniel, R. et al. Science (1993) 262:1546-1550. S. coelicolor protoplasts were transformed with pKW16 and stable thiostrepton resistant colonies were transferred to tap water medium agar plates containing 50 μg/ml thiostrepton.

(iii) Isolation and characterisation of (2S)-methyl-(3R)-hydroxypentanoic acid and (2S)-methyl-(3R)-hydroxybutanoic acid.

A colony of S. coelicolor CH999/pKW16 was picked and transferred to 100 ml YEME supplemented with 50 μg/ml thiostrepton and allowed to grow at 30°C. After 4 days the broth was filtered to remove mycelia, acidified to pH 3.0 and solid sodium chloride added until the solution was saturated. The broth was extracted 5 times with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were dried by extraction with saturated sodium chloride solution and concentrated by evaporation. Thin layer chromatography on silica gel plates, eluted with ethyl acetate:acetic acid 99:1 (v/v) and stained with potassium permanganate, showed the presence of a compound with the same mobility (Rf 0.55) as a reference sample of (2S)-methyl-(3R)-butanoic acid, which was not present in an extract obtained from S. coelicolor CH999 alone. Electrospray mass spectrometry (ESMS) analysis, in the negative ion mode, of the ethyl acetate extracts showed a major peak at m/e 117 not present in the control sample. In positive ion mode, and in the presence of formic acid, a peak was observed at m/e 119, which shifted to m/e 141 in the presence of added sodium ions. The exact mass of the sodium adduct was determined to be 141.05171 (the sodium salt of 2-methyl-3-hydroxybutanoic acid requires 141.05248). When a colony of S. coelicolor CH999/pKW16

was picked and transferred to 100 ml YEME supplemented with 50 μg/ml thiostrepton and allowed to grow at 30 °C for 7 days, an ethyl acetate extract prepared as above showed an additional peak, in ESMS operated in negative ion mode, at m/e 131. In ESMS operated in positive ion mode, and in the presence of added formic acid, the peak is found at m/e 155. The exact mass of this peak was determined to be 155.06973 (the sodium salt of 2-methyl-3-hydroxypentanoic acid requires 155.06890).

Example 18

Construction of plasmid pAR33

Plasmid pAR33 contains a hybrid Type I PKS comprising the ery loading module, extension module 1 of the ery PKS, extension module 12 of the rap PKS, and the ery chain-terminating thioesterase. It is constructed via several intermediate plasmids as follows (Figure 11):

Construction of plasmid pARRAP

The 4.7 kbp DNA segment of the rapC gene encoding module 12 of the rapamycin PKS was amplified by PCR employing the CloneAmp procedure (Raschtian, A. et al. Anal. Biochem. (1992) 91:91-97) and with the following two oligonucleotides as primers:

5'-ACGCGUACUAGUCAGATCTGGGCATCAATTCGCTGACCGCGGTGGAACTGCGCAA-3'

and

5'-AUGGAGAUCUCUCAGATCTTGAATGCGGCGGCTGCGGGGATGGTGCTGGCGTCA-3', and using as template the DNA of clone λ-1C (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843). Approximately 30-60 ng of the PCR product (4.7 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA (Gibco BRL), the mixture is cooled on ice and transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid (7.4 kbp) is identified by its restriction map and is designated pARRAP.

Construction of plasmid pAR32

Plasmid pARRAP is digested with BglII to release the 4.7 kbp fragment encoding rap module 12, which is purified by gel electrophoresis and then ligated into plasmid pKW15, which has been linearised by digestion with BglII. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid is one in which the rap module 12 has the correct orientation with respect to the coding sequence of the open reading frame of the insert in pKW15, so that a hybrid triketide lactone synthase gene is produced. Such a plasmid is identified by its restriction pattern, and is designated pAR32.

Construction of plasmid pAR33

Plasmid pAR32 contains an insert that can be excised by digestion with NdeI and XbaI, but there is an additional NdeI site in the insert that must be specifically protected against cleavage. This is done using the RecA protection method (Koob, M. et al. Nucl. Acids Res. (1992) 20:5831-5835)). The synthetic oligonucleotide 5'-GCACCCACGACGCCACCACCACATATGCCCTGCACCCTGCCCTCC-3' (in which the NdeI site is underlined) is used together with purified RecA protein and ATPγS, to form a stable triplex DNA-protein complex that specifically protects the internal NdeI site in rap module 12 from digestion. The protected plasmid pAR32 is digested with NdeI and XbaI, producing the desired full-length insert (13.1 kbp), and this is ligated with plasmid pRM52 (Example 4) which has been digested with NdeI and XbaI. The ligation mixture is transformed into E. coli TG1 recO and individual colonies are screened for their plasmid content. The desired plasmid pAR33 is identified by its restriction pattern.

Example 19

Construction of S. erythraea JC2/pAR33 and preparation of TKL derivatives

(i) Construction

Approximately 5 μg of plasmid pAR33 is transformed into protoplasts of S. erythraea JC2 and stable thiostrepton resistant colonies are selected. Total DNA from one such colony is isolated and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the chromosomal copy of the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase. This strain is designated S. erythraea JC2/pAR33.

(ii) Production of a novel triketide lactone by S. erythraea JC2/pAR33

S. erythraea JC2/pAR33 is inoculated into sucrose-succinate medium containing 50 μg/ml thiostrepton, and allowed to grow for five days at 28-30°C. After this time, the broth is filtered, and extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts are dried over anhydrous sodium sulphate and the ethyl acetate is removed by evaporation under reduced pressure. Electrospray MS of the residue showed the presence of Ac-2-nor-3-epi-TKL (III, R=Me) and 2-nor-3-epi-TKL (III, R=Et).

Example 20

Construction of plasmid pAR8

Construction of a hybrid triketide lactone synthase containing the ery loading didomain and ery chain-terminating thioesterase/cyclase, and modules 11 and 12 of the rap PKS

This example requires the initial construction of five separate plasmids, four housing separate elements of the target construct, and a fifth housing a gene conferring resistance to tetracycline. The inserts in these plasmids are sequentially combined by standard in vitro recombinant DNA techniques to form plasmid pAR5. A further three cloning steps lead to the final expression plasmid pAR8 (Figure 12).

Construction of plasmid pARLD

The segment of the ery AI gene from nucleotide 1 to nucleotide 1673, encoding the loading AT-ACP didomain, was amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUCCGATTAATTAAGGAGGACCATCAATGGCGGACCTGTCAAAGC TC-3' and

5'-AUGGAGAUCUCUCCGCTAGCGGTTCGCCGGGCGCCGCTTCGTTGGTCCGCGCGCG GGTTTCCC-3'

and plasmid pBK6.12 (Example 5) as template, to give plasmid pARLD.

Construction of plasmid pAR11

The segment of the rapC gene of S. hygroscopicus (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) from nucleotide 112 to nucleotide 2095, the 5'- end of the DNA encoding rap module 11, is amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-AUGGAGAUCUCUCCGCTAGCGATTGTGGGTATGGCG-3'

and

5'-ACGCGUACUAGUCCATGCATCTGCAGCACGGCGGCCTCATCACCGGA-3'

and the DNA of recombinant bacteriophage λ-1C (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as the template. Approximately 30-60 ng of the PCR product (2.0 kbp) is digested with uracil DNA glycosylase for 30 min at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1 recO and individual colonies checked for their plasmid content. The desired plasmid (4.7 kbp) is identified by its restriction map and is designated pAR11.

Construction of plasmid pAR12

The segment of the rapC gene of S. hygroscopicus

(Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) from nucleotide 7405 to nucleotide 9396, the 3' end of the DNA encoding rap module 12, is amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUCCATGCATTCCCGGAGCGGCGATCTGTGG-3'

and

5'-AUGGAGAUCUCUCCCGCGGCCGCGCTGTCACGCACCAGCTTC AGCAGTGCGTC-3' and the DNA of recombinant bacteriophage 1-1C (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as template. Approximately 30-60 ng of the PCR product (2.0 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and tranformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid (4.7 kbp) is identified by its restriction map and is designated pAR12.

Construction of pARTE

The 1.3 kbp segment of the eryAIII gene, extending by 132 nucleotides 3' of the eryAIII stop codon to a KpnI site, and encoding the C-terminal chain-terminating thioesterase/cyclase of DEBS, is amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUCCGCGGCCGCGATCCTCGGGCATTCCAGC-3'

and

5'-AUGGAGAUCUCUAAGCATTGGTAACTGTC-3', and plasmid pEXDB3 (Roberts, G. A. et al. Eur J. Biochem. (1993) 214:305-311) as the template. Approximately 30-60 ng of the PCR product (1.3 kbp) is digested with uracil DNA glycosylase for 30 min at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1 recO and individual colonies checked for their plasmid content. The desired plasmid (4.0 kbp) is identified by its restriction map and is designated pARTE.

Construction of plasmid pARTr

The 1.3 kbp segment of plasmid pBR322 containing the tetracycline resistance gene is amplified by the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUATCTAGACCATGCATGTTTGACAGCTTATCATC-3'

and

5'-AUGGAGAUCUCUATCTAGACCATGCATGCCGCCGGCTTCCATTCA-3'

and plasmid pBR322 as the template. Approximately 30-60 ng of the PCR product (1.3 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1recO and individual colonies

are checked for their plasmid content. The desired plasmid (4.0 kbp) is identified by its restriction map and is designated pARTr.

Construction of plasmid pAR1

Plasmid pARLD is digested with NheI and HindIII, and ligated to the 2.0 kbp NheI-HindIII insert obtained from plasmid pAR11. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR1.

Construction of plasmid pAR2

Plasmid pAR1 is linearised with NsiI and ligated with the NsiI fragment from pARTr. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR2.

Construction of plasmid pAR3

Plasmid pAR2 is digested with SpeI and XbaI and the insert is ligated with plasmid pAR12 which has been linearised with SpeI. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their

plasmid content. The desired plasmid is identified by its restriction map and is designated pAR3.

Construction of plasmid pAR4

Plasmid pAR3 is digested with NsiI and the vector is ligated to the NsiI fragment of pARTr, containing the tetracycline resistance gene. The ligation mixture is transformed into E. coli TG1recO and individual colonies, grown in the presence of tetracycline (12.5 μg/ml), are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR4.

Construction of plasmid pAR5

Plasmid pAR4 is digested with NotI and XbaI and ligated with a NotI-XbaI fragment obtained by digestion of plasmid pARTE. The ligation mixture is transformed into E. coli TG1recO and individual colonies, grown in the presence of tetracycline (12.5 μg/ml), are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR5.

Construction of plasmid pAR5-2

A 7.2 kbp segment of the rapC gene of S. hygroscopicus is excised from cosmid 13 (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) using BstXI and NdeI,

purified by gel electrophoresis, and ligated with plasmid pAR5 which has also been digested with BstXI and NdeI. The ligation mixture is transformed into E. coli TG1recO and individual colonies, grown in the presence of tetracycline (12.5 μg/ml), are checked for their plasmid content. The desired plasmid (11.9 kbp) is identified by its restriction map and is designated pAR5-2.

Construction of plasmid pAR5-3

A 3.0 kbp segment of plasmid pAR5 is excised by digestion with NdeI, purified by gel electrophoresis, and ligated with plasmid pAR5-2 which had been linearised with NdeI. The ligation mixture is transformed into E. coli TG1recO and individual colonies, grown in the presence of tetracycline (12.5 μg/ml), are checked for their plasmid content. The desired plasmid (14.9 kbp) is identified by its restriction map and is designated pAR5-3.

Construction of plasmid pAR8

A 12.2 kbp fragment of plasmid pAR5-3 is excised using PacI and XbaI, purified by gel electrophoresis, and ligated with plasmid pRM52 (Example 4) which had been cut with PacI and XbaI. The ligation mixture is transformed into E. coli TG1recO and and individual colonies, grown in the presence of tetracycline (30.3 μg/ml), are checked

for their plasmid content. The desired plasmid (14.9 kbp) is identified by its restriction map and is designated pAR8.

Example 21

Construction of S. erythraea JC2/pAR8 and production of TKL derivatives

(i) Construction

Approximately 5-10 μg pAR8, isolated from E. coli DH10B (pAR8) is used to transform S. erythraea JC2 protoplasts and stable thiostrepton resistant colonies are selected. One of these colonies is selected and total DNA is prepared for Southern hybridisation analysis, to confirm that the plasmid has integrated specifically into the chromosomal copy of the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase. This strain is designated S. erythraea JC2/pAR8

(ii) Production of 2,4-bisnor-3-epi-TKL and (Ac)-2,4-bisnor-3-epi-TKL

A colony of S. erythraea JC2/pAR8 is picked and transferred to sucrose-succinate medium supplemented with 50 μg/ml thiostrepton and allowed to grow at 30°C. After 3 days the broth is filtered and extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate

extracts are dried over anhydrous sodium sulphate and concentrated under reduced pressure. GC-MS of the residue shows the presence of 2,4-bisnor-3-epi-TKL (IV,R=E+) and (Ac)-2,4-bisnor-3-epi-TKL (IV, R=Me)

Example 22

Construction of plasmid pE1A2TE

Plasmid pE1A2TE (like plasmid pE1A2TE-2 also described herein) consists of a pT7.7 derived plasmid containing a hybrid Type I PKS gene comprising the ery loading module, the first extension module of the ery PKS, then the second extension module of the avr PKS, and the thioesterase of the ery PKS. It is constructed via several intermediate plasmids as follows (Figure 13).

Construction of plasmid pIG70

Plasmid pVE1446 which contains a portion of the avermectin PKS genes was obtained from E. coli ATCC 68250. Plasmid pVE1446 was digested with BamHI and the 7.0 kbp fragment between coordinates 6.05 and 13.05 was purified by gel electrophoresis and ligated into plasmid pUC119 which had

been linearised with BamHI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Of the two possible orientations of the BamHI insert pIG70 was selected such that when digested with PstI fragments of approximately 2.0 and 8.6 kbp were obtained and when digested with EcoRI fragments of approximately 5.1 and 5.5 kbp were obtained.

Construction of plasmid pIG71

Plasmid pVE1446 which contains a portion of the avermectin PKS genes was obtained from E. coli ATCC 68250. Plasmid pVE1446 was digested with BamHI and the 7.1 kbp fragment between coordinates 13.05 and 20.15 was purified by gel electrophoresis and ligated into plasmid pUC119 which had been linearised with BamHI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Of the two possible orientations of the BamHI insert pIG71 was selected such that when digested with EcoRI and XhoI 2 fragments of approximately 5 kbp were obtained.

Construction of plasmid pIG70ΔPst

pIG70 was cut with SacI and religated. pIG70ΔPst was isolated after transformation into E. coli TG1 recO.

Construction of plasmid pIG70ΔEco

pIG70 was cut with EcoRI and religated. pIG70ΔEco was isolated after transformation into E. coli TG1 recO

Construction of plasmid pIG71ΔSac

pIG71 was cut with SacI and religated. pIG71ΔSac was isolated after transformation into E. coli TG1 recO

Construction of plasmid pIGPCRstart

50 pmol of each of synthetic oligonucleotides 8985 (5'-GAGCAGTCGTTCCGAGATCTCGGCTTCGATTCA-3') which introduced a BglII site and 9204 (5'-GGGAGGAGATCAGATCCCAGAAGT-3') were used by PCR to amplify a 300 bp product from 60 ng pIG70ΔEco. The PCR product was end-repaired, phosphorylated and ligated into pUC18 that had been linearised with SmaI and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The orientation of pIGPCRstart was identified by a double restriction enzyme digest with EcoRI and BglII to give a pattern that included a 300 bp fragment.

Construction of plasmid pIGPCRend

50 pmol of each of synthetic oligonucleotides 8986 (5'-GAGGGAGTCGAACCGAGATCTCGGAACGCGCGG-3') which introduced a BglII site and 9205 (5'-GGGGGATCCTGGGGTCGGCCGGGCAGGGCAA-3') were used by PCR to amplify a 440 bp product from 60 ng pIG71ΔSac. The PCR product was end-repaired, phosphorylated and ligated into pUC18 that had been linearised with SmaI and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The orientation of pIGPCRend was identified by its restriction enzyme digest pattern.

Construction of plasmid pIGstart+middle

Plasmid pIGPCRstart was digested with PstI and the 300 bp fragment was purified by gel electrophoresis and ligated into plasmid pIG70ΔPst which had been linearised with PstI and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Plasmids which contained the correct orientation of the PstI-PstI insert were identified by DNA sequencing.

Construction of plasmid pIGAve2Bgl

Plasmid pIGstart+middle was digested with BamHI and the 5.0 kbp fragment was purified by gel electrophoresis and ligated into plasmid pIGPCRend which had been cut with BamHI and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Plasmids which contained the correct orientation of the BamHI-BamHI insert were identified by DNA sequencing.

Construction of plasmid pE1A2TE

Plasmid pIGAve2Bgl was digested with BglII and the 6 kbp fragment was purified by gel electrophoresis and ligated into plasmid pKW15 (Example 16) which had been linearised with BglII and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Plasmids which contained the correct orientation of the BglII-BglII insert were identified by restriction enzyme digest with EcoRI.

Example 23

Construction and use of plasmid pIG2

(i) Construction

Plasmid pE1A2TE was digested with NdeI and XbaI and the 11 kbp fragment was purified by gel electrophoresis and ligated into plasmid pRM52 (Example 4) which had been cut with NdeI and XbaI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content.

(ii) Construction of S. coelicolor CH999/pIG2

Plasmid pIG2 which had been isolated from E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) was transformed into protoplasts of S. coelicolor CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG2 was confirmed by its restriction pattern.

Example 24

Construction of plasmid pIG102

Plasmid pE1A2TE was digested with NdeI and XbaI and the 11 kbp fragment was purified by gel electrophoresis and ligated into plasmid pCJR101 (Example 2) which had been cut with NdeI and XbaI. The ligation mixture was used to

transform E. coli TG1 recO and individual colonies were checked for their plasmid content.

Example 25

Construction of plasmid pMO7

Plasmid pMO7 (like plasmid pMO107 also herein described) is an SCP2*-based plasmid containing a PKS gene comprising the ery loading module, the first and second extension modules of the ery PKS and the ery chain-terminating thioesterase, except that the DNA segment encoding the methylmalonyl-CoA:ACP acyltransferase within the first ery extension module has been specifically substituted by the DNA encoding the malonyl-CoA:ACP acyltransferase of module 13 of the rap PKS. It was constructed via several intermediate plasmids as follows (Figure 14).

Construction of plasmid pMO1

The approximately 1.3 kbp DNA segment of the eryAI gene of S. erythraea extending from nucleotide 1948 to nucleotide 3273 of eryAI (Donadio, S. et al. Science (1991) 252:675-679) was amplified by PCR employing as primers the synthetic oligonucleotides:

5'-CATGCTCGAGCTCTCCTGGGAAGT-3' and

5'-CAACCCTGGCCAGGGAAGACGAAGACGG-3', and plasmid pNTEP2 (Example 5) as template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO1 (3.9 kbp), in which the StuI site bordering the insert is adjacent to the HindIII site in the polylinker, was identified by its restriction pattern.

Construction of plasmid pMO2

The approximately 0.85 kbp DNA segment of the rapA gene of S. hygroscopicus, extending from nucleotide 1643 to nucleotide 2486 of rapA, was amplified by PCR employing as primers the following oligonucleotides:

5'-TTCCCTGGCCAGGGGTCGCAGCGTG-3' and
5'-CACCTAGGACCGCGGACCACTCGAC-3', and the DNA from the recombinant bacteriophage λ-1E (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as the template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO2 (3.5 kbp) was

identified by its restriction pattern.

Construction of plasmid pMO3

The approximately 1.7 kbp DNA segment of the eryAI gene of S. erythraea extending from nucleotide 4128 to nucleotide 5928 of eryAI, was amplified by PCR employing as primers the synthetic oligonucleotides:
5'-TGGCCAGGGAGTCGGTGCACCTAGGCA-3' and
5'-GCCGACAGCGAGTCGACGCCGAGTT-3' and plasmid pNTEP2 as template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO3 (4.4 kbp), in which the BalI and AvrII sites are adjacent to the HindIII site of the polylinker, was identified by its restriction pattern.

Construction of plasmid pMO4

Plasmid pMO1 was digested with HindIII and BalI and the 1.3 kbp insert was ligated with plasmid pMO3 which had been digested with HindIII and BalI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO4 (5.6 kbp) was identified by its

restriction pattern.

Construction of plasmid pMO5

Plasmid pMO4 was digested with StuI and the 3.0 kbp insert was ligated with plasmid pNTEP2 which had been digested with StuI and the vector purified by gel electrophoresis to remove the 3.8 kbp insert. The ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO5 (12.8 kbp) was identified by its restriction pattern.

Construction of plasmid pMO6

Plasmid pMO2 was digested with BalI and AvrII and the insert was ligated with plasmid pMO5 which had been digested with BalI and AvrII. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO6 (13.5 kbp) was identified by its restriction pattern.

Construction of plasmid pMO7

Plasmid pMO6 was digested with NdeI and XbaI and the insert was ligated with plasmid pRM52 (Example 4) which had been digested with NdeI and XbaI and purified by gel

electrophoresis. The ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO7 (also designated pRMAT2) was identified by its restriction pattern.

Example 26

Construction of S. coelicolor CH999/pMO7 and production of TKL derivatives

(i) Construction

Plasmid pMO7 which had been isolated from E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) was transformed into protoplasts of S. coelicolor CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pMO7 was confirmed by its restriction pattern.

(ii) Production and isolation of 4-nor-TKL and (Ac)4-nor-TKL using S. coelicolor CH999/pMO7

S. coelicolor CH999/pMO7 was inoculated into YEME medium containing 50 μg/ml thiostrepton and allowed to grow for five days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH 3. The broth was extracted twice with two volumes of ethyl acetate and the combined ethyl acetate extracts were

washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 200 mg crude product. This was digested with 2 ml of methanol, and mixed with 0.5 g of dry silica gel, and then subjected to flash chromatography on a column of the same material (1 cm x 15 cm) The column was eluted with diethyl ether, and fractions of 10 ml each were collected. Fractions 4-8 were pooled, and the diethyl ether was evaporated to leave about 10 mg of oily residue containing the compounds of interest. These were purified further by hplc on an octadecylsilica reverse phase column (10 mm x 25 cm) eluted at a flow rate of 2 ml/minute first with an isocratic mixture of water/methanol 75:25 (vol/vol) for five minutes, then with a linear gradient of increasing methanol, reaching water/methanol 55/45 (vol/vol) after 30 minutes. After about 11 minutes, fractions were collected containing, as the minor component, (Ac)4-nor-TKL (II, R_1=Me, R_2=H, R_3=Me) and after about 18 minutes fractions were collected containing, as the major component, 4-nor-TKL (II, R_1=Me, R_2=H, R_3=ET).

The ^1H spectrum of 4-nor-TKL was determined using a Bruker AM-400 NMR spectrometer. Found: δH (400 MHz, $CDCl_3$) 4.18 (1H, dtd, 11.8, 6.1, 2.9 Hz, H-5), 3.75 (1H, ddd, 11.0, 10.0, 4.0 Hz, H-3), 2.35 (1H, dq, 10.0, 7.0 Hz, H-2), 2.20 (1H, ddd, 13.3, 4.0, 2.9 Hz, H-4eq), 1.6 - 1.88 (3H, m, 2xH-6, H-4ax), 1.41 (1H, d, 7.0 Hz, CH3-3'), 1.01 (1H, t,

7.5 Hz, CH3-7) ppm.

The ^{13}C NMR spectrum of 4-nor-TKL was also determined (100 MHz, $CDCl_3$): 173.3 (C-1), 77.7 (C-5), 70.4 (C-3), 45.1 (C-2), 37.7 (C-4), 28.8 (C-6), 13.5 (C-3'), 9.1 (C-7).

Example 27

Construction of plasmid pMO107 and production of TKL derivatives

(i) Construction

Plasmid pMO6 was digested with NdeI and XbaI and the insert was ligated with plasmid pCJR101 (Example 2) which had been digested with NdeI and XbaI and purified by gel electrophoresis. The ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO107 was identified by its restriction pattern.

(ii) Production and isolation of 4-nor-TKL and (Ac)4-nor-TKL using S. erythraea JC2/pMO107

S. erythraea JC2/pMO107 was prepared by standard techniques (c.f. Example 26(i)) and inoculated into sucrose-succinate medium containing 50 μg/ml thiostrepton and allowed to grow for three-five days at 28-30°C. After

this time the broth was filtered to remove mycelia and the pH adjusted to pH 3. The broth was extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 10 mg/L crude product. This was digested with 2 ml of methanol, and mixed with 0.5 g of dry silica gel, and then subjected to flash chromatography on a column of the same material (1 cm x 15 cm) The column was eluted with diethyl ether, and fractions of 10 ml each were collected. Fractions 4-8 were pooled, and the diethyl ether was evaporated to leave about 15 mg of oily residue containing the compounds of interest. These were purified further by hplc on an octadecylsilica reverse phase column (10 mm x 25 cm) eluted at a flow rate of 2 ml/minute first with an isocratic mixture of water/methanol 75:25 (vol/vol) for five minutes, then with a linear gradient of increasing methanol, reaching water/methanol 55/45 (vol/vol) after 30 minutes. After about 11 minutes, fractions were collected containing, as the minor component, (Ac)4-nor-TKL and after about 18 minutes fractions were collected containing, as the major component, 4-nor-TKL.

The ^{1}H and ^{13}C spectra of the purified 4-nor-TKL and (Ac)4-nor-TKL were identical with the spectra obtained for authentic material.

Example 28

Construction of S. erythraea ERMD1, carrying a hybrid PKS gene in which the avr loading didomain is substituted for the ery loading didomain of S. erythraea NRRL 2338

(i) Construction of plasmid pAVLD

Plasmid pCRabc (Example 9) was linearised with BamHI and ligated to pIJ702 previously digested with BglII. The mixture contained the desired plasmid pAVLD (Figure 15). The ligation mixture was transformed into E.coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pAVLD was identified by its restriction pattern (Figure 15).

(ii) Construction of *S.erythrea ERM D1*

Approximately 5-10 mg of pAVLD, isolated from E. coli TG1recO(pAVLD) was transformed into S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. One of these colonies was selected and total DNA was digested with PstI and analysed by Southern hybridisation employing as a probe the insert from plasmid pCRc which contains the fragment of the ery AI gene encoding the ketosynthase domain KS1. The analysis showed positively-hybridizing PstI fragments of 8.5 kbp, 4.8 kbp and 33 kbp, indicating the presence of two tandemly integrated copies of pAVLD (Figure 16).

Example 29

Isolation of erythromycins altered at C-13

A 50 ml fermentation of S. erythraea ERMD1 was carried out on tap water medium and after 4 days at 30°C the mycelium was harvested and used to inoculate 1.5 litres of sucrose-succinate medium containing thiostrepton (50μg/ml).

After growth at 30°C for 4 days, the whole broth was extracted twice with an equal volume of ethyl acetate.

The combined extracts were concentrated under reduced pressure and subjected twice to preparative thin layer chromatography on silica plates (20 x 20cm) eluted with chloroform/methanol/.88 ammonia 8:2:0.01 (by vol). The products were separated by hplc on a PhaseSep C18 base-deactivated reverse phase column S5odS (octadecylsilica) 6(4.6mm x 250mm), eluted with methanol/0.5% ammonium acetate (70:30 (vol/vol), at 1 ml.min. Fractions were collected between 7 and 11 minutes from three separate injections, and the pooled fractions were re-injected in ten separate injections. The order of elution from the column was: erythromycin B analogues, followed by erythromycin D analogues and erythromycin A analogues. B and D analogues emerged after 8-10 minutes, erythromycin A analogue 3-4 minutes later. The analogues containing a C-4 (isobutyryl) starter unit are eluted earlier, with the

analogues with C-5 (isovaleryl) starter unit emerging several minutes later, although the C-4 late (eryA analogue) and the early C-5 (erythromycins B and D analogue) overlap. High resolution MS gave results for C-4 eryA, eryB and eryD analogues, and for C-5 eryA and eryB analogues, which correspond closely to those calculated:

Analogue	Calc'd Mass	Measured Mass
C5-eryA	762.5004	762.5021
C4-eryA	748.4847	748.4820
C5-eryB	746.4898	748.5077
C4-eryB	732.4898	732.4933

In these experiments natural erythromycins were present only in low or undetectable amounts, and there were no detectable amounts of eryC analogues. The overall concentration ratio of C-4/C-5 compounds in the fermentation broth, as assessed by ESMS of ethyl acetate extracts of broths, was between 4:1 and 6:1 in favour of C-4 compounds. The ratio of A:B:D analogues is variable, about 15:60:25, but with an increasing proportion of A analogues as the fermentation proceeds. The total yield of erythromycins is about 400 μg/litre.

Example 30

Construction and use of S. erythraea NRRL2338/pRMTE

(i) Construction

Approximately 5 μg of plasmid pRMTE (Example 6) isolated from E. coli TGI recO (pRMTE) was transformed into protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. One of these was selected and designated S. erythraea NRRL2338/pRMTE.

(ii) Enhanced production of erythromycin A and erythronolides using S. erythraea NRRL2338/pRMTE

S. erythraea NRRL2338/pRMTE was grown in sucrose and succinate medium containing 50 μg/ml thiostrepton at 28-30°C. After 3 days the whole broth was extracted twice with an equal volume of ethyl acetate, the combined ethyl acetate extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. Examination of the extract by thin layer chromatography on silica plates eluted with isopropyl ether:methanol:ammonium hydroxide 75:35:1 (by volume) showed the presence of several components. Electrospray mass spectrometry of the extracts revealed the presence of a mixture of erythromycin A, erythronolide B (EB) and 6-deoxyerythronolide B (6-DEB), together with minor amounts of (Ac)-6-DEB as its sodium adduct, (Ac)-EB as its

Na adduct (411.1), and EB as its Na adduct (424.1), and also TKL (m/e 159.1). The yield of erythromycin A plus erythronolide B was about 500 mg/L of medium, compared to about 50 mg /L produced by S. erythraea NRRL2338 fermented under identical conditions. Cells of S. erythraea NRRL2338/pRMTE harvested from the fermentation broth after 3 days were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Three high molecular weight bands, corresponding to the erythromycin PKS multienzyme subunits DEBS1, DEBS2 and DEBS3 were observed, approximately ten times more intense than the same protein band seen from cell extracts of S. erythraea NRRL2338 prepared by the same procedure (Caffrey, P. et al. FEBS Letters (1992) 304:225-228).

An identical fermentation of S. erythraea NRRL2338/pRMTE was carried out except that the medium was supplemented with 5 mM potassium propionate. After three days the broth was extracted with ethyl acetate as before, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and concentrated. Preparative TLC using the system isopropyl ether:methanol:ammonium hydroxide 75:35:1 (by volume) separated two major components. Analytical TLC showed that the faster running component (Rf 0.8) has the same mobility as authentic 6-DEB; and the slower migrating material was an approximately equal mixture of a component of Rf 0.63, co-migrating with an

authentic sample of TKL; and a component of Rf 0.60, with the same mobility as an authentic sample of EB. Electrospray mass spectrometry (ESMS) on a VG BioQ mass spectrometer operated in positive ion mode showed that the component of Rf 0.75 had m/e 387.4, as required for 6-DEB. ESMS of the mixture of the components with Rf values 0.60 and 0.63 confirmed the presence of TKL and EB.

Example 31

Construction and use of S. erythraea TER43/pRMTE

(i) Construction

Approximately 5 mg of plasmid pRMTE was transformed into protoplasts of S. erythraea TER43 (Cortes, J. et al.,Science (1995) 268:1487-1489) and stable thiostrepton resistant colonies were isolated. One of these was selected and designated S. erythraea TER43/pRMTE.

(ii)Enhanced production of TKL using S. erythraea TER43/pRMTE

S. erythraea TER43/pRMTE was inoculated into 1L sucrose-succinate medium and allowed to grow for 3 days at 28-30°C. After 3 days, the broth was extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate and concentrated. Analysis of the extract by electrospray

mass spectrometry (operated in the positive ion mode) showed the presence of TKL (m/e 173.1) and of (Ac)-TKL (m/e 159.1). The combined yield of triketide lactones was 100 mg/L, compared with 10 mg/L obtained by fermentation of S. erythraea TER43 under identical conditions. Cells of S. erythraea TER43/pRMTE, harvested from the fermentation broth after 3 days, were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. A high molecular weight band, corresponding to the erythromycin PKS subunit DEBS1 with the attached thioesterase domain (Cortes, J. et al. Science (1995) 268:1487-1489) was observed, approximately ten times more intense than the same protein band seen from cell extracts of S. erythraea prepared by the same procedure.

Example 32

Construction and use of S. erythraea NRRL2338/pCJRTE

(i) Construction

Approximately 5 μg of plasmid pCJRTE is transformed into protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the eryA genes by homologous recombination, so as to place the resident eryA genes

under the control of the actI promoter derived from plasmid pCJRTE, while the DEBS1-TE gene borne by the incoming plasmid is placed by the integration event under the control of the chromosomal eryA promoter.

(ii) Enhanced production of erythromycins and their precursors using S. erythraea NRRL2338/pCJRTE.

S. erythraea NRRL2338/pCJRTE is inoculated into sucrose-succinate medium containing 50 mg/ml thiostrepton and allowed to grow for four days at 30°C. After this time the broth is filtered to remove mycelia and then extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extracts are analysed by mass spectrometry and it is found that the mixture contains erythromycin A, accompanied by 6-DEB, (Ac)-DEB, TKL and (Ac)-TKL, in total amounts 100 mg/L, or 5 times the total amount of erythromycins and precursors of erythromycins that are obtained using S. erythraea NRRL2338 under the same conditions.

Example 33

Construction and use of S. erythraea JC2/pCJRTE

(ii) Construction

Approximately 5 μg of plasmid pCJRTE is transformed into protoplasts of S. erythraea JC2 and stable thiostrepton

resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Enhanced production of triketide lactones using S. erythraea JC2/pCJRTE

S. erythraea JC2/pCJRTE is inoculated into sucrose-succinate medium containing 50 μg/ml thiostrepton and allowed to grow for four days at 30°C. After this time the broth is filtered to remove mycelia and then extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extracts are analysed by mass spectrometry and NMR and it is found that the major product is TKL, and the minor product (Ac)TKL, in total yields (100 mg/L) 10 fold greater than obtained using S. erythraea TER43.

Example 34

Construction and use of S. erythraea NRRL2338/pIG1

(i) Construction

Approximately 5 μg of plasmid pIG1 is transformed into

protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Production of 14-membered lactones using S. erythraea NRRL/pIG1

S. erythraea NRRL/pIG1 is inoculated into tap water medium containing 50 μg/ml thiostrepton and allowed to grow for four days at 30°C. After this 20 ml of the mycelium is used to seed 500 ml of sucrose-succinate medium containing 50 mg/ml thiostrepton, in a 2L flask with a single spring to reduce clumping, shaken at 280 rpm. After between 3.5 and 6 days, the broth is filtered to remove mycelia and then extracted three times with a quarter volume of ethyl acetate. The combined ethyl acetate extracts are dried over anhydrous sodium sulphate and solvent removed by evaporation. Analysis of the product mixture using GC and electrospray MS revealed that of a total of 5-6 mg/L of 14-membered macrolide products, the major component was (s-pent)-erythromycin D (about 1.5 mg/L), with other components present being (s-pent)-erythromycin B and (s-pent)-erythromycin A; (i-but)-erythromycins A, B and D; and small amounts of

natural erythromycins A, B and D. The extracts also contained significant amounts (11 mg/l) of TKL's: (s-pent)-TKL (5mg/l), (i-but)-TKL and TKL. (NB s-pent and i-but indicate 1-methylpropyl and isopropyl side-chains, respectively, corresponding to the use of s-pentanoyl and i-butanoyl starter substrates.)

Example 35

Determination of antibiotic activity of novel erythromycin A analogues

A 3 ml overnight culture of *Bacillus subtilis* ATCC 6633 was grown at 30°C in nutrient broth (Difco). 200ml of nutrient 1.5% agar (difco) at 46°C was seeded with 1ml of the *B.subtilis* culture and poured immediately into petri dishes (25 ml/plate). After drying the plates in a laminar flow hood for 15 minutes, wells (0.4mm in diameter) were cut using a cork borer and 20 microlitres of the test compound as a solution in ethanol (5-10 mg/L) was added to each well. The plates were kept at 4°C for 5-7 hours to allow the compound to diffuse, and the plates were then incubated overnight at 30°C. Clear zones of growth inhibition were seen with both (i-but)-and (s-pent)- erythromycin A.

Although the present invention is illustrated by the

examples listed above, they should not be regarded as limiting the scope of the invention. The above descriptions illustrate first, how a specific promoter for a Type II PKS gene set, coupled to its specific cognate activator gene, contrary to expectation, may be used to achieve controlled and enhanced expression of Type 1 PKS genes in a heterologous host. Examples of these hosts that are given are S. erythraea and S. avermitilis, but it will be evident to those skilled in the art that alternative hosts, drawn from a wide range of actinomycetes, will equally well serve as expression hosts. Similarly, although the actI promoter and its cognate activator gene actII-orf4 have been used in these Examples, it will be evident to those skilled in the art that other Type II PKS promoter/activator gene combinations are well-known and characterised which will be equally efficacious in directing the controlled and enhanced expression of Type 1 PKS genes in heterologous cells drawn from a wide range of actinomycetes. Examples of such promoter/activator gene combinations include the promoters of the dnr gene cluster and the dnrI activator gene from the daunorubicin gene cluster of Streptomyces peucetius: (Madduri, K. and Hutchinson, C.R. J. Bacteriol (1995) 177:1208-1215) and the promoter of the gene redX and the activator gene redD from the undecylprodigiosin gene cluster of S. coelicolor (Takano, E. et al. Mol. Microbiol. (1992) 2: 2797-2804).

Secondly, the above descriptions illustrate for the first time the construction of hybrid Type I PKS genes and their use to obtain novel polyketide products of utility as chiral synthetic intermediates or as bioactive materials such as antibiotics. Hybrid PKS genes have been constructed either by substitution of loading modules, or by substitution of individual domains in extension modules; or by substitution of whole modules. Thus, the replacement of the ery loading module by the avr loading loading module has been described herein to obtain either novel erythromycin A analogues or triketide lactones. It will readily occur to those skilled in the art that other alterations of the ery loading module can be obtained through its replacement with the loading module of other Type I PKS gene sets. Examples of such alterations include replacement with the loading module of the rap PKS; and with the loading module of the FK506-producing PKS. Such alterations will lead to the synthesis of polyketides specifically altered in their starter unit.

It is well-known to those skilled in the art that the avr loading module is capable of accepting a wide range of non-natural carboxylic acids as alternative starter units, when these are included in the fermentation medium. Therefore in the light of the present invention, it is evident that in addition to the synthesis of novel erythromycin A derivatives in which the C-13 substituent is isopropyl or sec-butyl instead of ethyl, which has been

shown here, many other novel erythromycin A derivatives can be obtained by feeding of the appropriate non-natural carboxylic acids (or compounds convertible to them by fermentation) to an appropriate strain housing the hybrid PKS, such non-natural carboxylic acids having in general the formula R-COOH, where R is an alpha-branched group, and where the carbon bearing the -COOH group is also attached to at least two other atoms or groups other than hydrogen, with the preferred non-natural carboxylic acids being those described for the production of non-natural avermectins in European Patent EP 214,731, March 18 1987, Pfizer). The resulting novel analogues of erythromycin A can be converted, by procedures well understood in the art, into further novel semi-synthetic derivatives of erythromycin A, of considerable utility in the treatment of bacterial infection, including for example ketolides and azalides. These embodiments of the invention are novel chiral materials of potential utility in the chemical synthesis of valuable bioactive products. The products which are 14-membered macrolides are novel erythromycin A analogues which are highly valuable antibacterial agents having the same microbial targets as do the known erythromycins and the semi-synthetic derivatives of known erythromycins, such as the ketolides disclosed in French patents Nos. 2697523 (06/05/94) Roussel Uclaf; 269724 (06/05/94) Roussel Uclaf; and 2702480 (16/09/94) Roussel Uclaf.

It will be evident to those skilled in the art that the replacement of the ery loading module by the loading module of the rap PKS will also lead to novel and useful analogues of erythromycin A, in which the natural propionate starter unit is substituted by a cycloalkylcarboxylic acid starter unit. Further examples of the formation of such hybrid Type I PKS include, but are not limited to, the replacement of the rap loading module in Streptomyces hygroscopicus by the avr loading module, leading to the formation of non-natural rapamycins; and the replacement of the avr loading module in Streptomyces avermitilis by the rap loading module, leading to the formation of further examples of non-natural avermectins. The present invention also encompasses mutants in which more than one of the genetic manipulations described in the examples are combined.

In the light of the present invention, it will also be evident that alterations in the specificity of the loading module of a Type I PKS can alternatively be achieved by the mutation of the genes encoding the natural loading module, and then selection for the desired altered specificity, as practised for example in the technique of in vitro gene shuffling (Stemmer, W. P. Nature (1994) 370:389-391).

The examples listed above also teach the construction and use of a low copy number plasmid vector pCJR101 as a

vector for delivery of PKS genes into suitable actinomycete hosts. Plasmid pCJR101 is derived from the plasmid SCP2* (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1977) 154:155-166) found in the strain Streptomyces coelicolor M110 deposited for example at the Northern Regional Research Laboratory, Peoria, Illinois, USA under the accession number NRRL 15041. Plasmid SCP2* has been previously used in the construction of several useful vectors such as pIJ2839 (Ingram, C. et al. J. Bacteriol. (1989) 171:6617-6624); plasmid pHJL197 (Larson, J. L. and Hershberger, C. L. J. Bacteriol. (1983) 157:314-317) and pRM5 (McDaniel, R. et al. Science (1993) 262:1546-1550). It will be evident to those skilled in the art that either these or other SCP2*-based plasmids may be substituted for pCJR101 either directly or after modification of the vector to introduce suitable promoter linked to the PKS genes, as demonstrated by the use of plasmid pRM5 in several Examples described herein. High copy number vectors such as plasmid pGM8 (Muth, G. et al. Mol. Gen. Genet. (1989) 219:341-350) derived from the Streptomyces ghanaensis plasmid pGS5 are also suitable as substitutes for pCJR101, as are integrative vectors such as plasmid pSAM2 (Murakami, T. et al. J. Bacteriol. (1989) 171:1459-14??). Those skilled in the art will readily appreciate the versatility of approaches to increasing the rate of biosynthesis of natural or non-natural complex polyketides such as macrolides and polyethers through heterologous use of type II PKS activator genes and their

cognate promoters as disclosed here, in numerous derivatives of vectors well known in the art as useful for genetic engineering in actinomycetes.

In the construction of hybrid type I PKS genes, the Examples teach how the structural genes encoding both donor and acceptor PKS components may be spliced together to create functional catalysts capable of bringing about the synthesis of novel polyketides. The present invention shows that in choosing where the junction will be made between the donor and the acceptor DNA it is, surprisingly, not necessary to limit the choice to positions known or predicted to lie between domains in so-called linker regions. Instead it is preferred for junctions to be in the edge regions of domains (particularly KS or AT domains), where the sequences are highly conserved. Further, creation of junctions that lead to conservative changes in amino acid sequence at such junctions in the gene product are tolerated. It is also evident that for the purposes of creating a hybrid, PKS modules may be combined from two or more natural PKS. In the examples given here, donor DNA is spliced into the acceptor DNA at a position variously in the acyl carrier protein (ACP) domain or in the ketosynthase (KS) domain of a module, but the scope of the invention includes hybrid PKS where the junctions between homologous domains are chosen to lie within any of the constituent parts of a type I PKS module. However, it will be found most

advantageous to select a position for each junction that lies within a domain, and close to one edge, so that the specificity of the chimaeric module is readily predictable, and so that disturbance of its proper functioning is minimised.

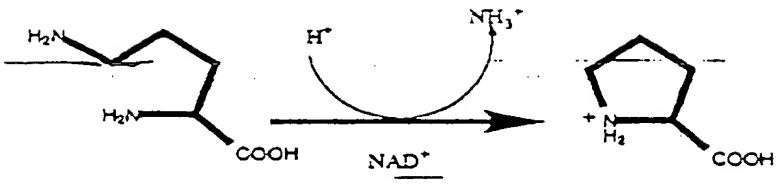
It will be readily appreciated that in the light of the present invention a hybrid PKS can be constructed by selecting pieces of DNA encoding respectively a loading module, a variable number of extension modules up to at least six in number, and a chain-releasing thioesterase domain; and concatenating the DNA, using standard procedures, in the order in which it is intended that the gene products operate. NB: The hybrid PKS with (say) 6 modules may be part of an assembly of synthases leading to a product produced by many more than 6 extension modules. It will also readily occur to those skilled in the art that the module-sized DNA fragments may be constituted in more than way, Thus the present invention includes the construction of functional hybrid PKSs exemplified by the construct containing the following activities in a single polypeptide chain:

AT0-ACP0-KS1-[ATR1-DHR1-ERR1-KRR1-ACPR1-KSR2]-AT2-KR2-ACP2-TE

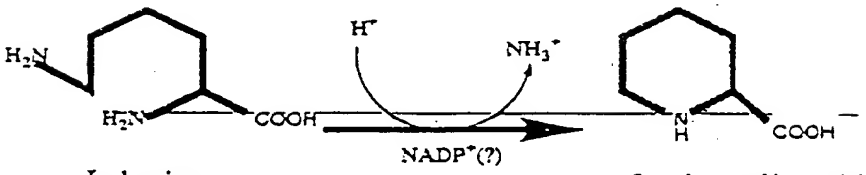
where the activities shown in square brackets are derived from modules 1 and 2 of the rap PKS, and the rest are

derived from the loading module, extension modules 1 and 2, and the chain-terminating thioesterase of DEBS1. In such constructs, each ketossynthase domain is kept together with the ACP, AT and reductive domains of the module that precedes it in the naturally occurring PKS from which it was derived, rather than with the activities of its own module. Alternative but equally functional arrangements of the module-sized DNA building blocks for construction of hybrid PKS will readily occur to those skilled in the art.

We have already mentioned the technique of suppressing the synthesis of a natural precursor in order to facilitate incorporation of a desired variant. Thus S.hygroscopicus contains a gene cluster for the synthesis of rapamycin. This cluster includes a gene (rapL) whose product is proposed to catalyse the formation of the rapamycin precursor L-pipecolate through the cyclodeamination of L-lysine (Molnar *et al.*, 1996). The conversation of L-lysine to L-pipecolic acid has been demonstrated in intact plants and rats and is observed as well with their extracts (Meister *et al.* 1957). An entirely analogous reaction to the production of L-pipecolate from L-lysine is the conversion of L-ornithine to L-proline which involves the deamination of the α-amino group prior to cyclisation (Muth and Costilow, 1974).



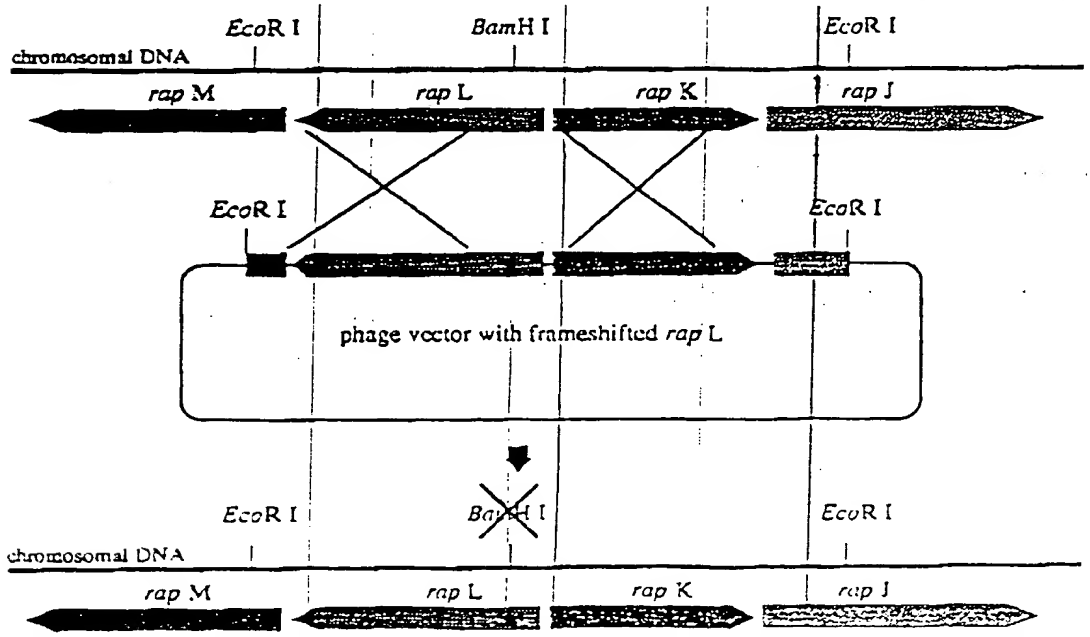
L-ornithine L-proline



L-lysine L-pipecolic acid

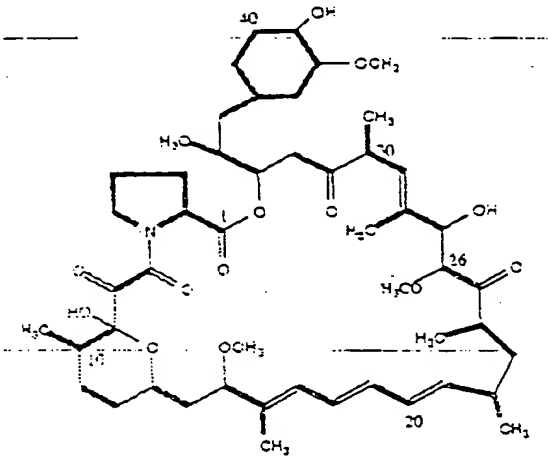
The construction of a *rapL* mutant.

In order to determine whether the lysine cyclodeaminase gene is involved in the biosynthesis of rapamycin as a precursor feeder, a chromosomal mutant of *S.hygroscopicus* was created by phage ΦC31-mediated gene replacement using the method of Lomovskaya *et al.* [Microbiology (UK) 1997]. A unique *Bam*H I site was found 42bp into the *rapL* gene(1032bp long). This *Bam*H I site was removed by end-filling with *E. coli* DNA polymerase I thus creating a frameshift in the *rapL* gene. A 3 kb *Eco*R I fragment encompassing the entire *rapL* gene flanked by *rapK* and part of the *rapM* genes respectively was cloned into the phage vector, KC515. The recombinant phage was used to transfect *S. hygroscopicus*. A double recombination event resulted in the creation of a chromosomal mutant of *S. hygroscopicus* with a frameshift in *rapL*.



Feeding studies

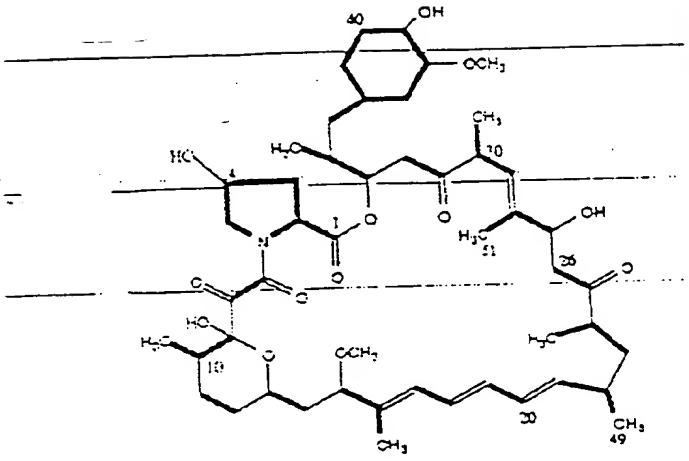
The mutant strain did not produce significant amounts of rapamycin. Feeding studies of the mutant with L-pipecolic acid or L-proline give rise to efficient production of rapamycin and prolyrapamycin respectively. Analogues of pipecolic acid were also fed to the mutant strain, in order to generate novel rapamycins.



Prolylrapamycin

Compound fed	Incorporation	Mass [m/z, M+Na⁺]	Retention time LC-MS (min)	Main Product
L-pipecolic acid	Yes	936	8.84	rapamycin
L-proline	Yes	922	7.99	prolyl-rapamycin
L-trans-4-hydroxyproline	Yes	938/908	5.35/6.29	4-hydroxy-prolyl-rapamycin and 4-hydroxy-prolyl-26-demethoxy-rapamycin
L-cis-4-hydroxyproline	Yes	938/908	5.35/6.29	as above
L-cis-3-hydroxyproline	Yes	938/908	5.35/6.29	3-hydroxy-prolyl-rapamycin and 3-hydroxy-prolyl-26-demethoxy-rapamycin
picolinic acid	No			
pyrrole-2-carboxylic acid	No			

Table 1



position	^{1}H δ [ppm]	^{13}C δ [ppm]
1		171.30 (169.2)
2	5.24 (5.29)	58.17 (51.3)
3	2.65, 1.69 (2.74, 1.76)	38.48 (27.0)
4	4.38 (1.78, 1.47)	70.63 (20.6)
5	3.37, 2.94 (1.75, 1.48)	56.53 (25.3)
26	3.58 (3.71)	not determined
27	3.89 (4.17)	70.63 (77.3)
38	2.93 (2.93)	83.90 (84.4)
39	3.37 (3.37)	73.95 (73.9)
40	1.99, 1.33 (1.99, 1.38)	31.22 (31.3)
49	3.12 (3.13)	55.68 (55.8)
51	3.39 (3.41)	56.50 (56.5)

Table 2

A selection of ^{1}H and ^{13}C NMR data for 4-hydroxy-26-demethoxyrapamycin and rapamycin (shifts for equivalent carbon positions indicated in brackets, McAlpine *et al.*)

e main product obtained from feeding the mutant with L-trans-4-hydroxyproline was purified and subjected to mass spectrometry .gmentation and nuclear magnetic resonance analyses. These analyses clearly suggest that the novel rapamycin produced is 4-hydroxyprolyl-26-demethoxyrapamycin.

Materials and Methods

Materials. All molecular biology enzymes and reagents were from commercial sources. Viomycin was a gift from Pfizer. L-pipecolic acid, L-proline, 3,4-dehydroproline, picolinic acid, pyrrole-2-carboxylic acid, trans 4-hydroxyproline, cis 4-hydroxyproline and cis 3-hydroxyproline were obtained from Aldrich Chemical Company.

Bacterial strains, phages and growth conditions

Escherichia coli DH10B (GibcoBRL) was grown in 2xTY medium as described by Sambrook *et al.* (1989). Vector pUC18 was obtained from New England Biolabs. *E. coli* transformants were selected with 100 mg/ml ampicillin. The rapamycin producer *Streptomyces hygroscopicus* NRRL 5491 and its derivatives were maintained on SY agar (Soluble starch 1.5%; yeast extract 0.1%; K_2HPO_4 0.1%; $MgSO_4$ x 7 H_2O 0.1%; NaCl 0.3%; N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) buffer 30 mM, pH7.4; agar 1.5%), and cultivated in Tryptic Soy Broth with 1.0% glucose, 100mM MES pH6.0, supplemented with 10 µg/ml viomycin when required. *S. lividans* JI1326 was cultivated in YEME (Hopwood *et al.*, 1985) or Tap Water Medi (0.5% glucose; 1% sucrose; 0.5% tryptone; 0.25% yeast extract; 36mg EDTA; pH 7.1). Liqu cultures were grown at 30°C in Erlenmeyer flasks with shaking at 200-250 rpm. Infection with the *att* actinophage KC515 (Chater, 1986) and its derivative *ΦΔRapL* (present work) were done on solid DNA medium supplemented with 10mM $MgSO_4$, 8mM $Ca(NO_3)$ and 0.5% glucose (Hopwood *et al.*, 1985).

Isolation and invitro manipulation of DNA

DNA manipulations, PCR and electroporation procedures were carried out as described in Sambrook et al. (1989). Total *S. hygroscopicus* DNA was isolated using the Gibco genomic DNA isolation kit

Southern hybridizations were carried out with probes labelled with digoxigenin using the DIG DNA labelling kit (Boehringer Mannheim). DNA fragments for labelling and subcloning were isolated with the Qiaex (Qiagen) gel extraction kit.

Construction of ΦΔ*RapL* carrying a frameshift in the *rapL* gene for homologous recombination in *S. hygroscopicus* .

pUC3*Eco*RI was constructed by cloning a 3034bp *Eco* RI fragment (nucleotide 93956 to 96990 of the *rap* cluster) encompassing the entire *rapL* gene flanked by *rapK* and part of the *rapM* genes respectively [redacted] into an *Eco* RI - cut pUC18 modified vector where the *Bam*HI site in the polylinker region has been removed. A unique *Bam* HI site (starting at nucleotide 95036 of the *rap* cluster) was found 42bp into the rapL gene (nucleotide 95078 to 94047 of the *rap* cluster;1032bp long) . Plasmid pUC3*Eco* RI was digested with *Bam* HI and the cohesive ends were filled in by treating it with *E.coli* DNA polymerase I (Klenow fragment). The ligated plasmid DNA was redigested with *Bam* HI and used to transform *E. coli.* Ampicillin resistant transformant were selected and their plasmid DNA checked for the removal of the *Bam* HI site by restricion enzyne analysis. This was confirmed by DNA sequencing. The 3kp insert was excised from the plasmid with *Eco* RI and the cohesive ends blunt-ended by treatment with *E.coli* DNA polymerase I (Klenow fragment). The blunt-ended insert was cloned into *Pvu* II cut phage vector KC515, resulting in ΦΔ*RapL* .

Protoplasts of *S. lividans* JI1326 were transfected with the phage construct as described by Hopwood *et al.* (1985). Recombinant phage was identified using PCR analysis. Infection of *S. hygroscopicus* NRRL 5491 with ΦΔ*RapL* was done according to Lomovskaya *et al.* (1996) on DNA plates supplemented with glucose, $MgSO_4$ and $Ca(NO_3)$. Lysogens were selected by overlaying the plates with 50 μg ml^{-1} (final concentration) viomycin 24 h post-infection. Strains that had undergone a second recombination event deleting the integrated phage were identified by selecting viomycin sensitive isolates after three rounds of non-selective growth and sporulation on SY plates. The insertion and subsequent loss of the phage were confirmed by genomic Southern hybridizations.

Precursor feeding and fermentation of *S. hygroscopicus* ΔRapL

Precursor feeding of *S. hygroscopicus* ΔRapL was performed routinely in 500 ml flasks containing 100ml of Tryptic Soy Broth with 1.0% glucose, 100mM MES pH6.0, supplemented with the appropriate pipecolic acid analogue, at a final concentration of 1mg/ml. *S. hygroscopicus* ΔRapL was also cultivated in 2l flasks containing 400 ml of chemically-defined media as described by Cheng *et al.* (1995). For large scale fermentation,10 µl of spores of *S. hygroscopicus* ΔRapL was used to innocule a 100 ml flask containing 30 ml of Tryptic Soy Broth medium. The flask was incubated on a rotary shaker (300 rpm) at 28°C for 4 days. 4 ml of the first seed culture was transferred to a 2l flask (second seed culture) containing 400 ml of the medium and incubated on a rotary shaker (300 rpm) at 28°C for 4 days. The second seed culture was transferred to a 20 l fermentor containng 15 l of the medium. Trans 4-hydroxyproline was added to the medium aseptically to a final concentration of 1mg/ml. The fermentation was carried out at 28°C for 4 days, with an agitation rate of 500 rpm. The cells were harvested and extracted with twice its volume of methanol overnight.

Purification and analysis of rapamycin and its derivatives

After 3-4 days fermentation mycelia were collected by filtration and extracted with two volumes of methanol at room temperature for 1 h. This crude extracts were analysed by lc-ms using a Finnigan MAT (San Jose, CA) LCQ with a Hewlett Packard (Böblingen, Germany) 1100 HPLC.
The large scale fermentation was worked up similarly. The crude extract was evaporated to dryness and then purified by flash chromatography (Merck silica gel 60, no. 9385) with acetone/hexane 1/1. The fractions containing rapamycins were further purified by preparative HPLC on a 250 x 20 mm RP18 column (HPLC Technology, Macclesfield, UK) using standard conditions. The 15 l fermentation yielded about 15 mg of pure prolyl-rapamycin and 3 mg of 4-hydroxy-prolyl-39-demethoxy-rapamycin. NMR were aquired on a Bruker DRX 500 spectrometer.

Biological activity of rapamycin analogues.

Rapamycin induces a specific cell cycle arrest in G1 in the cell line 536. 536 is a human B lymphocytic line immortalised by Epstein Barr virus infection. The potency of each analogue was compared to that of rapamycin using the 536 cells as

a bioassay. The 536 cells (obtained from the human genetic mutantn cell repository, Camden, New Jersey, USA) were cultured in Iscoves medium supplemented with 10% fetal calf serum. For bioassay, 536 cells were seeded into 96 well microtitre plates at 10,000 per well in 100µl of growth medium. Drug stocks of 1mM in DMSO were prepared and further dilutions were made to give a constant final concentration of 0.1% DMSO in growth medium. Control cultures were treated with 0.1% DMSO in growth medium; experimetal cultures received a final concentration of 10^{-7}M, 10^{-8}M, 10^{-9}M or 10^{-10}M rapamycin or rapamycin analogue. Each culture was set up in triplicate and replicate plates were labelled with 1µCi tritiated thymidine (Amersham International, specific activity 70Ci/mM) per well for 3h at either 0h; 24h; or 48h incubation with drugs. At the respective time points the cultures were harvested onto glass fibre paper to trap the DNA following water lysis: free nucleotides were washed away. Radioactivity incorporated into the filter discs / trapped DNA was counted in a Packard scintillation counter using biodegradable scintillation fluid.

Results

Characterisation of a frameshift chromosomal mutation in the *rapL* gene

To confirm that the *rapL* gene product is indeed involved in the biosynthesis of rapamycin as a precursor feeder, the frameshift chromosomal mutant *S.hygroscopicusΔRapL* was isolated as described in Materials and Methods. This mutation was investigated by Southern blot hybridisation using the 3kb *Eco*RI fragment (93956 - 96990) to probe *Bgl* II/*Bam* HI digested chromosomal DNA (data not shown). Analysis of the wild-type *S.hygroscopicus* shows the expected 5.9kb (representing nucleotides 89118 - 95036) and 2.7kb *Bam* HI/*Bgl* II fragments (representing nucleotides 95036 - 97710) after hybridisation. When chromosomal DNA of *S.hygroscopicusΔRapL* was treated similarly, only a 8.6kb *Bam* HI/*Bgl* II fragment (representing nucleotides 89118 - 97710) was detected, indicating that the *Bam* HI site at position 95036 has been removed. This was confirmed by PCR analysis. Chromosomal DNA was subjected to PCR using oligonucleotides primers identical to, respectively, the sequences from nucleotide 93950 to 93968; and from 96990 to 97010. The expected 3kb DNA fragment was amplified from wildtype DNA and following *Bam*III digest, two bands roughly 2kb and 1kb in size were detected. In samples containing *S.hygroscopicusΔRapL* chromosomal DNA the 3kb PCR product amplified was found to be resistant to *Bam*HI digest (data not shown).

Precursor feeding of the chromosomal mutant *S.hygroscopicus ΔRapL*

Growing cultures of the mutant *S.hygroscopicus* ΔRapL were fed with different amino acid precursors (table 1). Only the three proline derivatives were found to be incorporated as judged by LC-MS. The main rapamycin derivative in the fermentations apart from prolyl rapamycin is a compound with m/z 908 which could correspond to a hydroxy-rapamycin lacking a methoxy group. Smaller amounts of a compound with m/z 938 were also detected which would correspond to hydroxy-prolyl-rapamycin. MS-fragmentation experiments (table 3) as well as the characteristic UV spectra clearly indicated that these compounds are rapamycin derivatives with a hydroxyproline incorporated. In order to get enough material for NMR characterisation we fed hydroxyproline on a

large scale to the mutant (15 L broth) and isolated 3 mg of the compound with m/z 908 as described in material and methods. The NMR data (table 2) showed the chemical shifts and couplings expected for the hydroxy-proline spin system. The changed chemical shifts for the positions 26 and 27 and the unchanged shifts for positions 38-40 as compared to rapamycin proved that the methoxy group is missing at position 26. MS-fragmentation data (table 3) confirmed these findings. This can be inferred from the loss of the C15-C26-fragment leading to a fragment with m/z 644 for both of the new rapamycin derivatives. Furthermore, the loss of the C28-C42-fragment (322 amu) can be seen for both compounds as well as for rapamycin, indicating that there is no modification in this part of the molecules. The ions at m/z 807 and 777 respectively which are equivalent to the loss of the amino acid (131 amu) confirm the presence of OH-proline. This means that the compound with m/z 908 is 4-hydroxy-26-demethoxy-rapamycin and the compound with m/z 938 is 4-hydroxyprolyl-rapamycin.

Biological activity

The dose response of human lymphoblastoid cell lines 536 In the experiment shown in Figure the mean cpm of radiolabelled thymidine incorporated into the untreated controls shows that 0-3 h drug exposure had no appreciable effect on DNA synthesis upto 100nM of rapamycin, prolylrapamycin, or 4-hydroxy-prolyl-26-demethoxy- rapamycin. This infers that none of the compounds were toxic to the 536 cell line. After 24 and 48 hours, the 536 cells showed a concentration-dependent inhibition of DNA synthesis with an ID50% of 1nM for rapamycin; and 3nM for prolylrapamycin. 4-hydroxy-prolyl-26-demethoxy- rapamycin was also inhibitory but did not reach 50% at 100nM. Previous experiments have shown that rapamycin is a profound inhibitor of G1 progression in the 536 cell line (Metcalfe *et al*. Oncogene 1997, I hope!). This is also suggested in these experiments for the rapamycin analogues, since no significant effect was found at 3h but inhibition was observed once the cell population had time to proceed through a complete cell cycle (24h) and reach the drug arrest point.

Rapamycin m/z	4-hydroxyprolylrapamycin m/z	4-hydroxyprolyl-26-demethoxyrapamycin m/z
936	938	908
904	906	876
807 (loss of pipecolate, 129 amu)	807 (loss of hydroxyproline, 131 amu)	777 (loss of hydroxyproline, 131 amu)
642	644	644
614	616	586
596	598	568
582	584	554
564	566	536

Table 3 : The electrospray mass spectrometry fragmentation patterns of rapamycin (m/z 936), 4-hydroxyprolyl-rapamycin (m/z 938) and 4-hydroxyprolyl-27-demethoxy-rapamycin (m/z 908); given are the m/z values of the sodium adducts.

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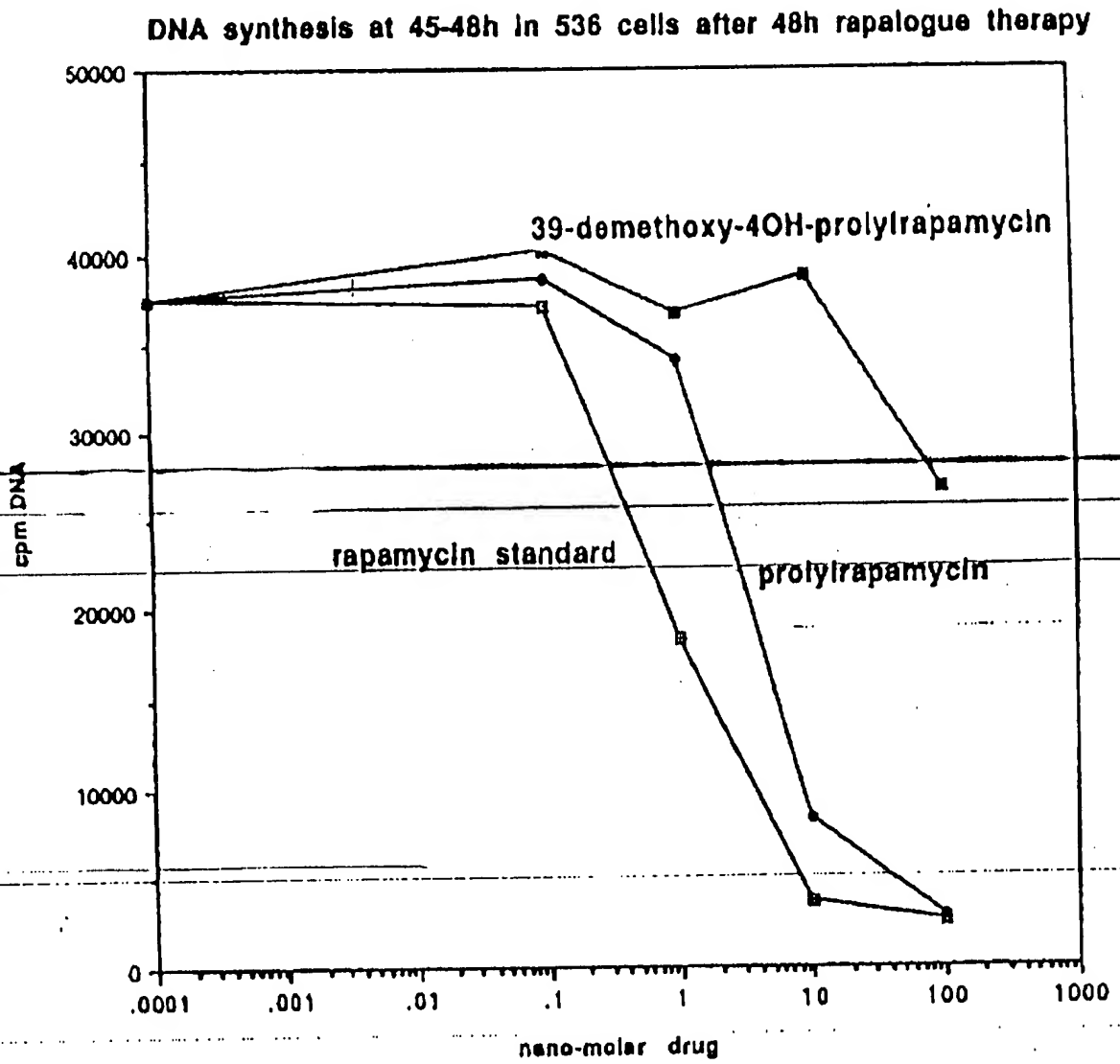
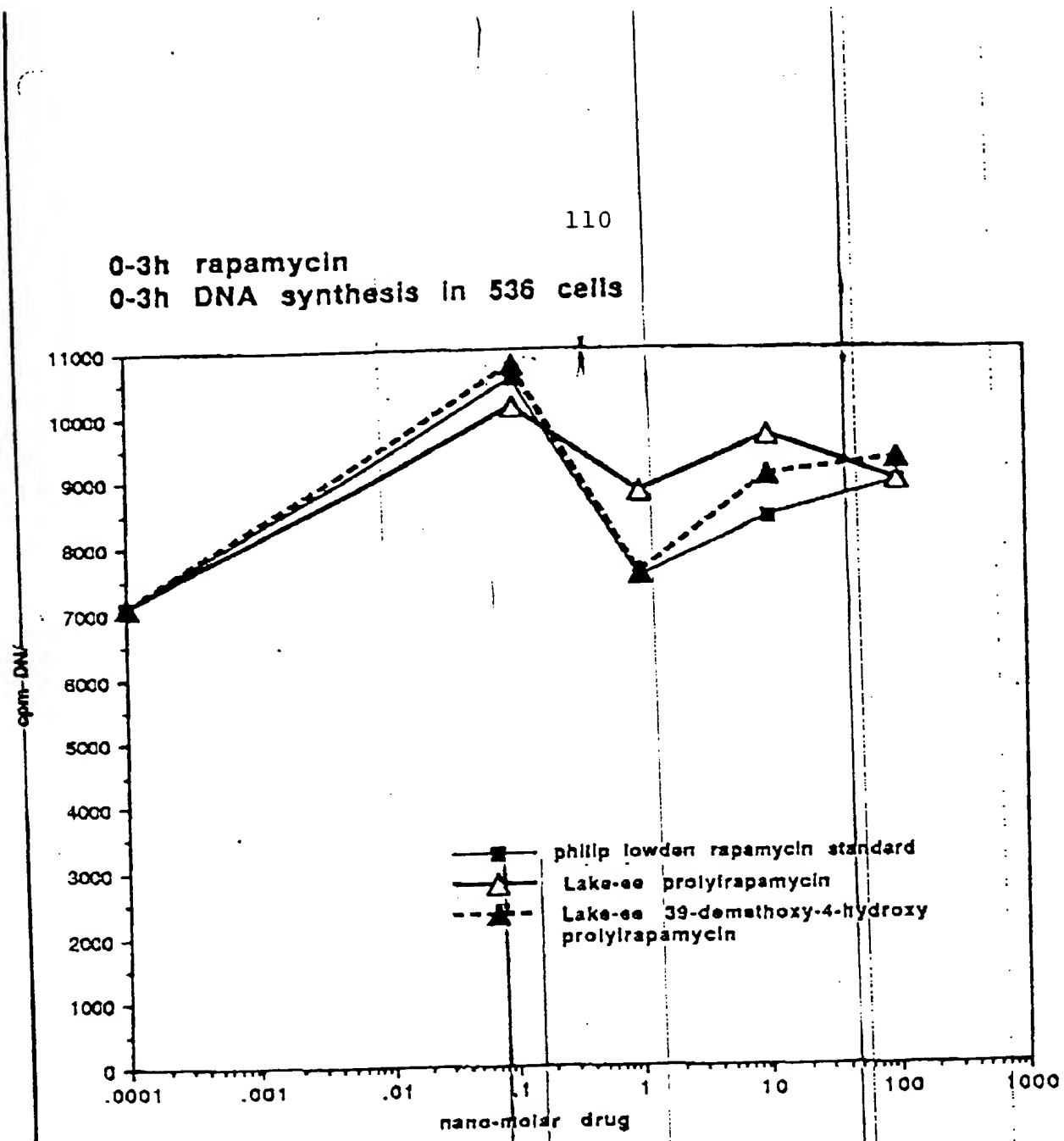
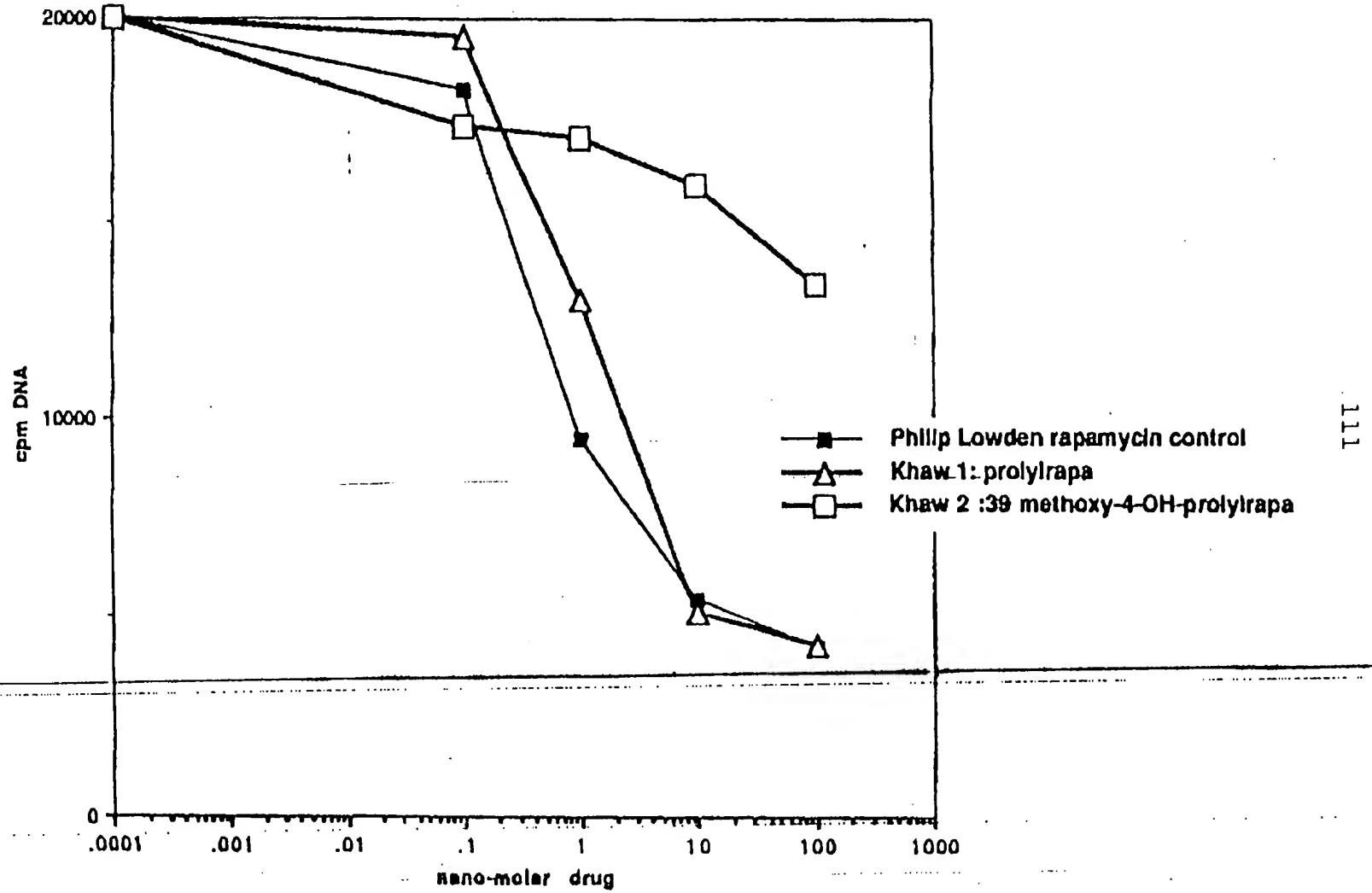



0-3h rapamycin
0-3h DNA synthesis in 536 cells
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cpm- DNA
.0001
.001
.01
.1
1
10
100
1000
nano-molar drug
philip lowden rapamycin standard
Lake-ee prolylrapamycin
Lake-ee 39-demethoxy-4-hydroxy prolylrapamycin

24h drug therapy; DNA synthesis of 536 cells 21-24h. Khaw rapalogues



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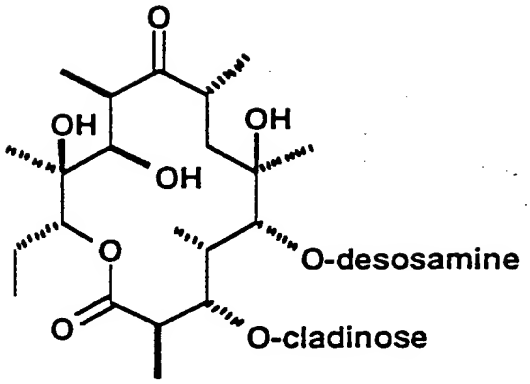
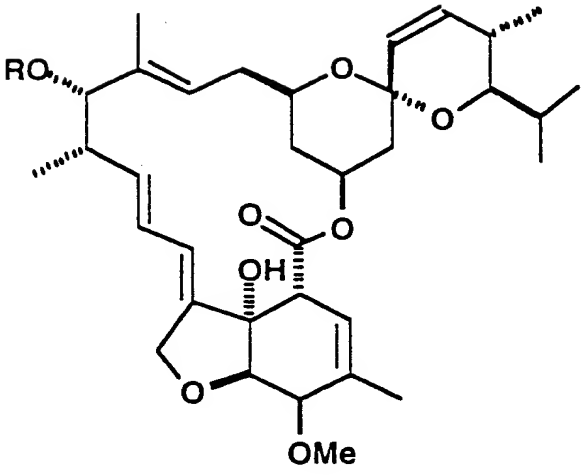
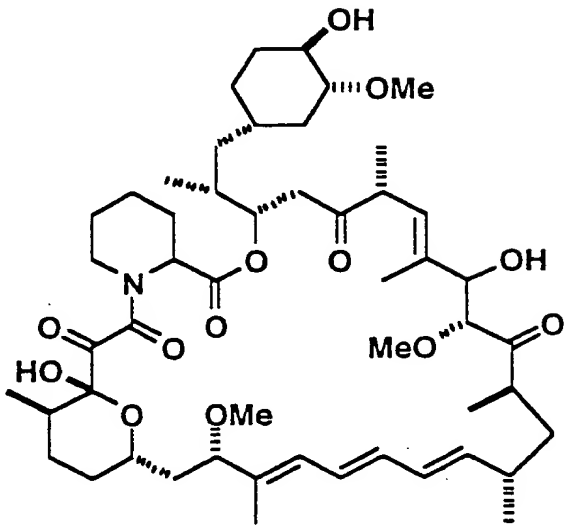


Erythromycin A



Avermectin A1b



Rapamycin

Figure 1.

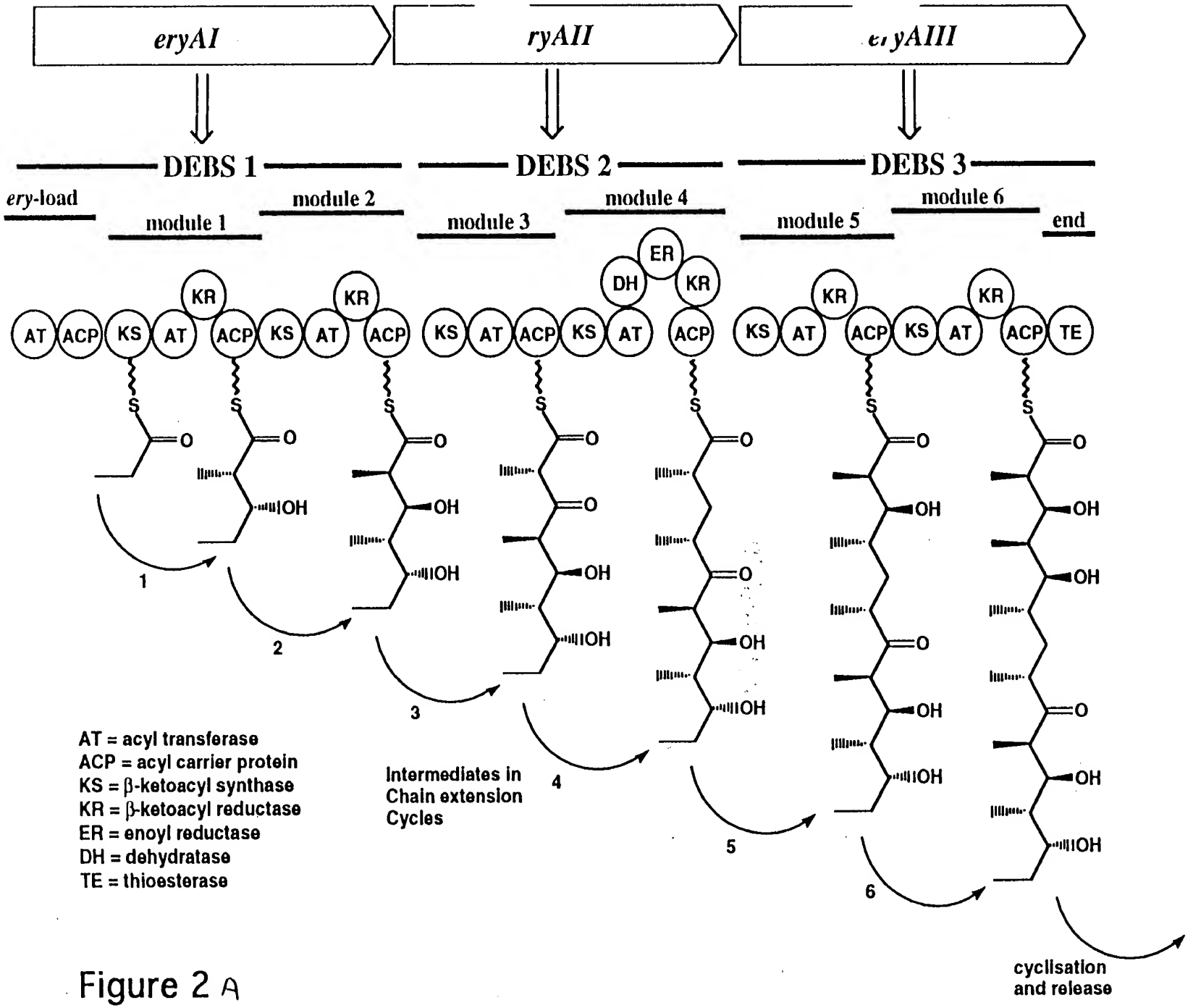
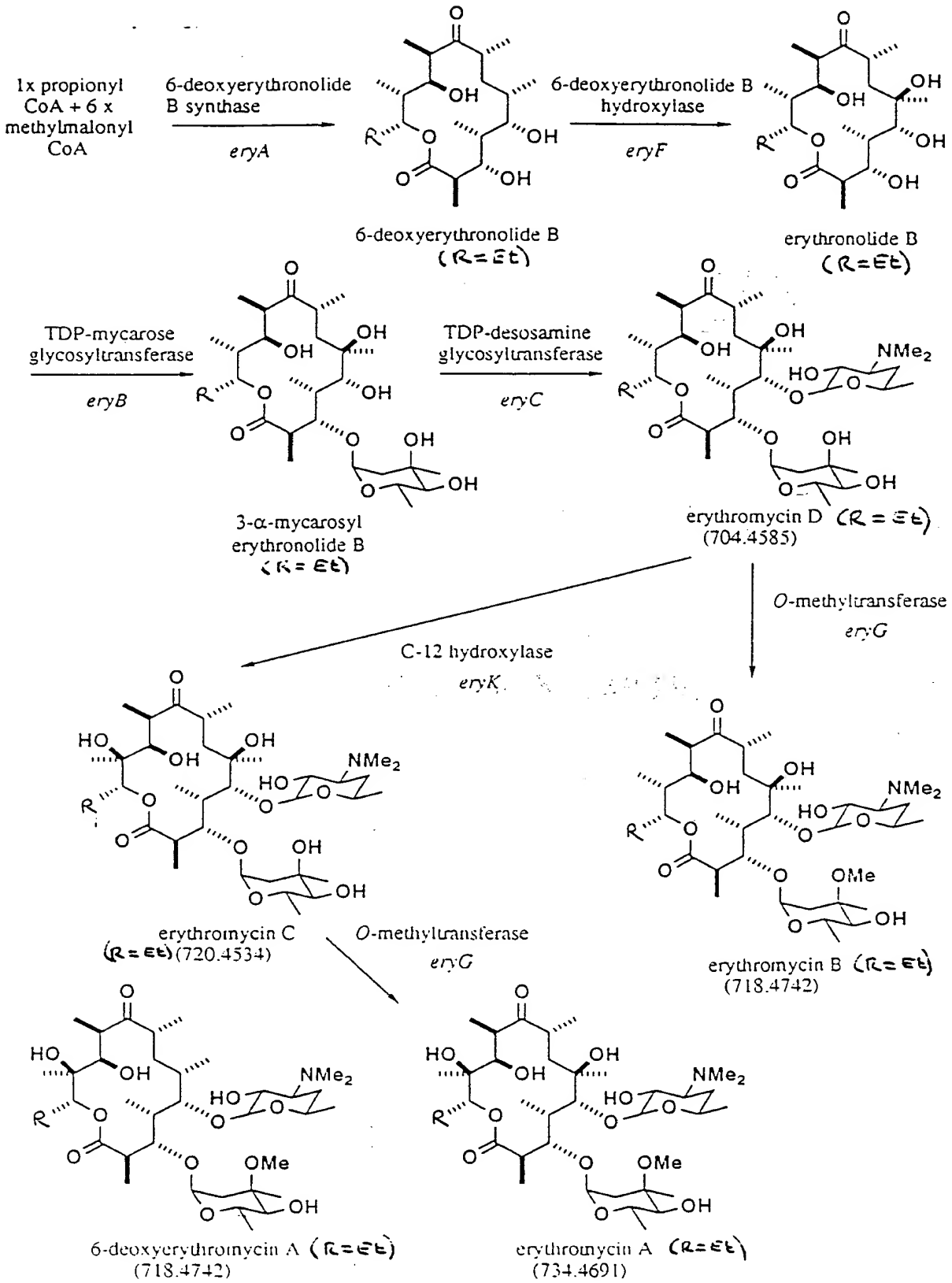

eryAI
ryAII
eryAIII
DEBS 1
DEBS 2
DEBS 3
ery-load
module 1
module 2
module 3
module 4
module 5
module 6
end
AT
ACP
KS
KR
DH
ER
TE
OH
1
2
3
4
5
6
AT = acyl transferase
ACP = acyl carrier protein
KS = β-ketoacyl synthase
KR = β-ketoacyl reductase
ER = enoyl reductase
DH = dehydratase
TE = thioesterase
Intermediates in Chain extension Cycles
cyclisation and release

Figure 2 A



Post-PKS biosynthesis of erythromycins

Fig 2B

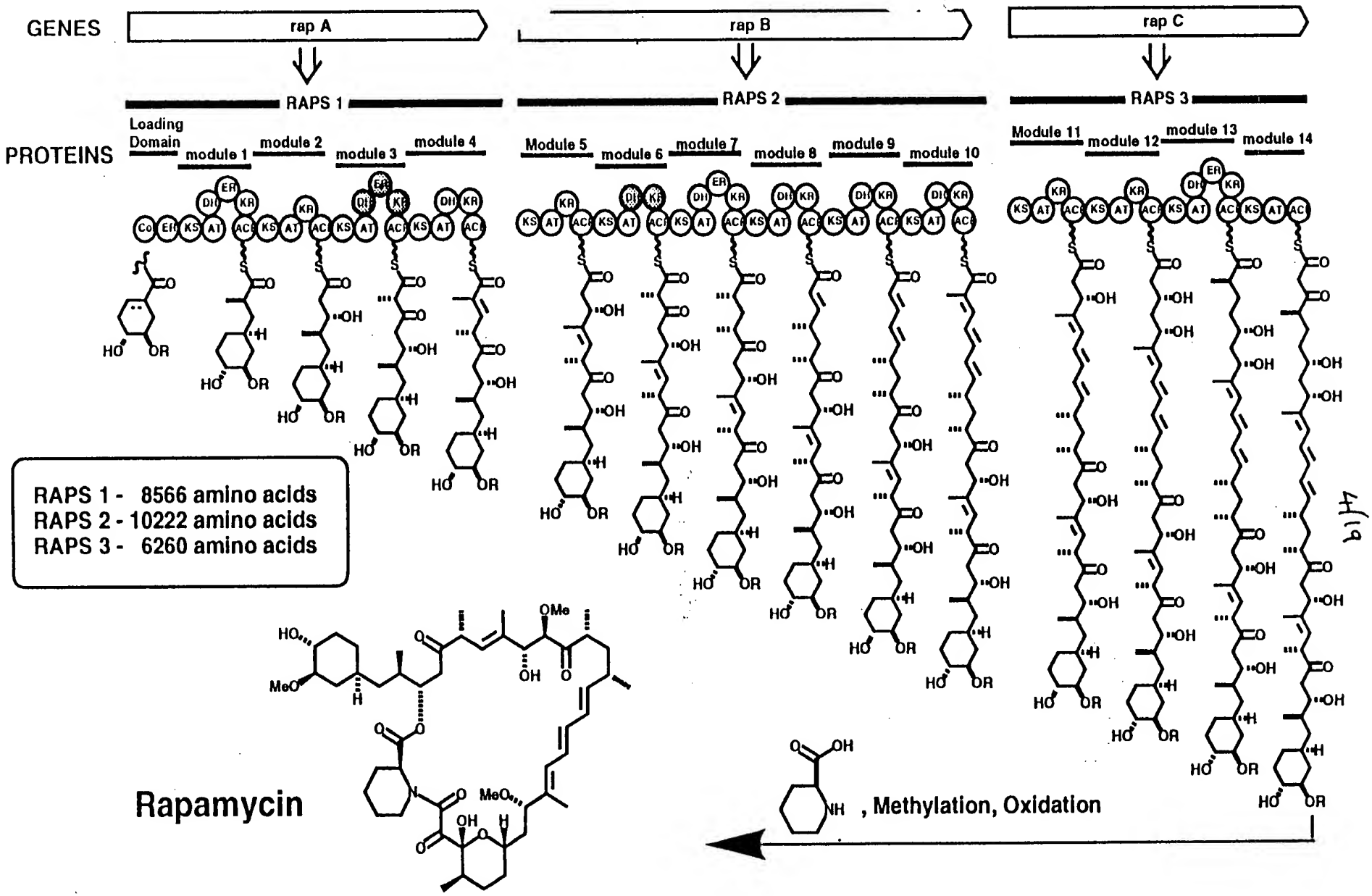

GENES
rap A
rap B
rap C
RAPS 1
RAPS 2
RAPS 3
PROTEINS
Loading Domain
module 1
module 2
module 3
module 4
Module 5
module 6
module 7
module 8
module 9
module 10
Module 11
module 12
module 13
module 14
RAPS 1 - 8566 amino acids
RAPS 2 - 10222 amino acids
RAPS 3 - 6260 amino acids
Rapamycin
, Methylation, Oxidation

Figure 3

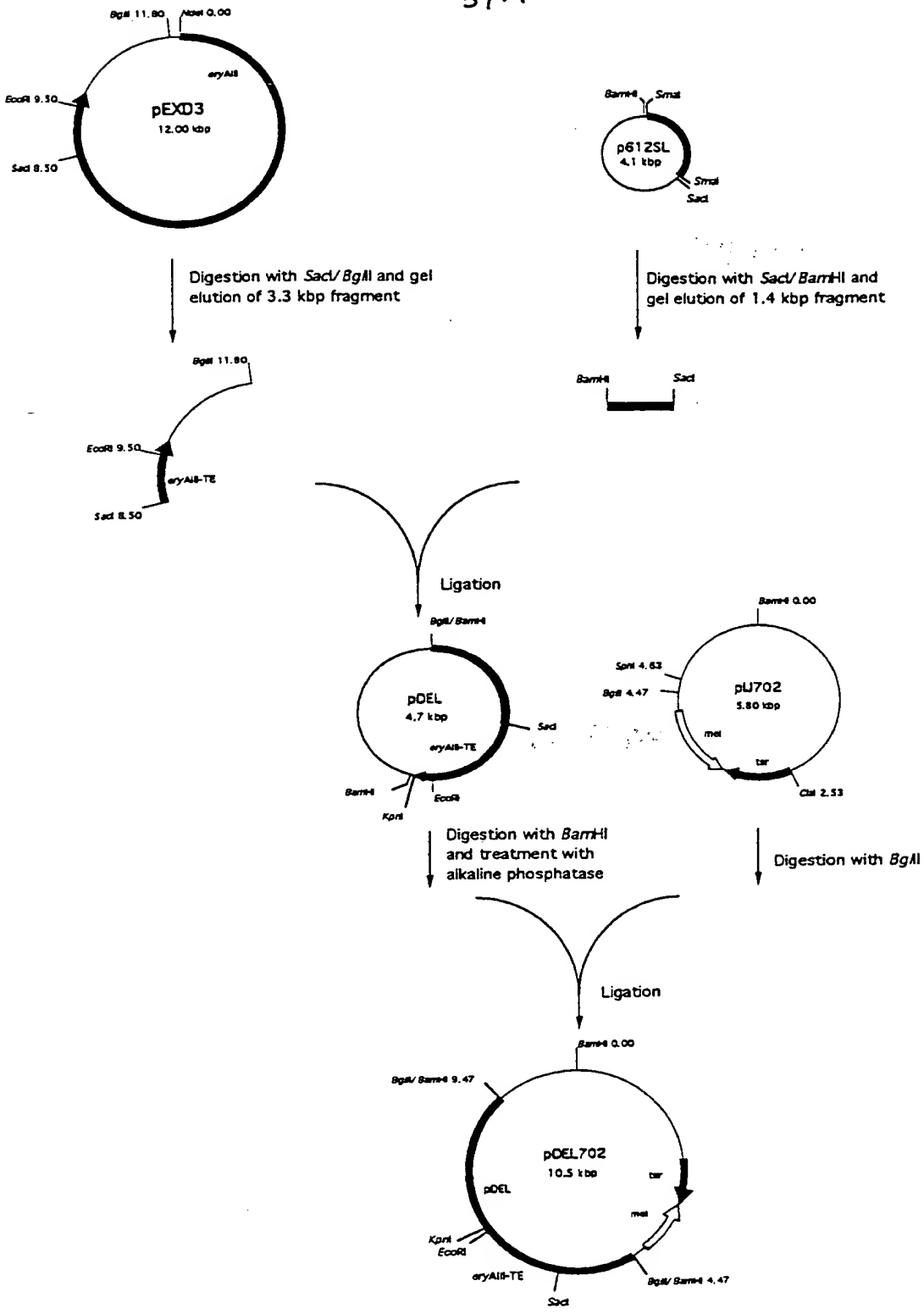

pEXD3
12.00 kbp
eryAI8
EcoRI 9.50
SacI 8.50
p612SL
4.1 kbp
BamHI
SmaI
SacI
Digestion with SacI/BglII and gel elution of 3.3 kbp fragment
Digestion with SacI/BamHI and gel elution of 1.4 kbp fragment
BglII 11.80
EcoRI 9.50
eryAI8-TE
SacI 8.50
BamHI
SacI
Ligation
BglII/BamHI
pOEL
4.7 kbp
SacI
eryAI8-TE
BamHI
EcoRI
KpnI
pIJ702
5.80 kbp
SphI 4.63
BglII 4.47
mel
tsr
ClaI 2.53
Digestion with BamHI and treatment with alkaline phosphatase
Digestion with BglII
Ligation
BamHI 0.00
BglII/BamHI 9.47
pOEL702
10.5 kbp
pOEL
tsr
mel
KpnI
EcoRI
eryAI8-TE
SacI
BglII/BamHI 4.47

Figure 4

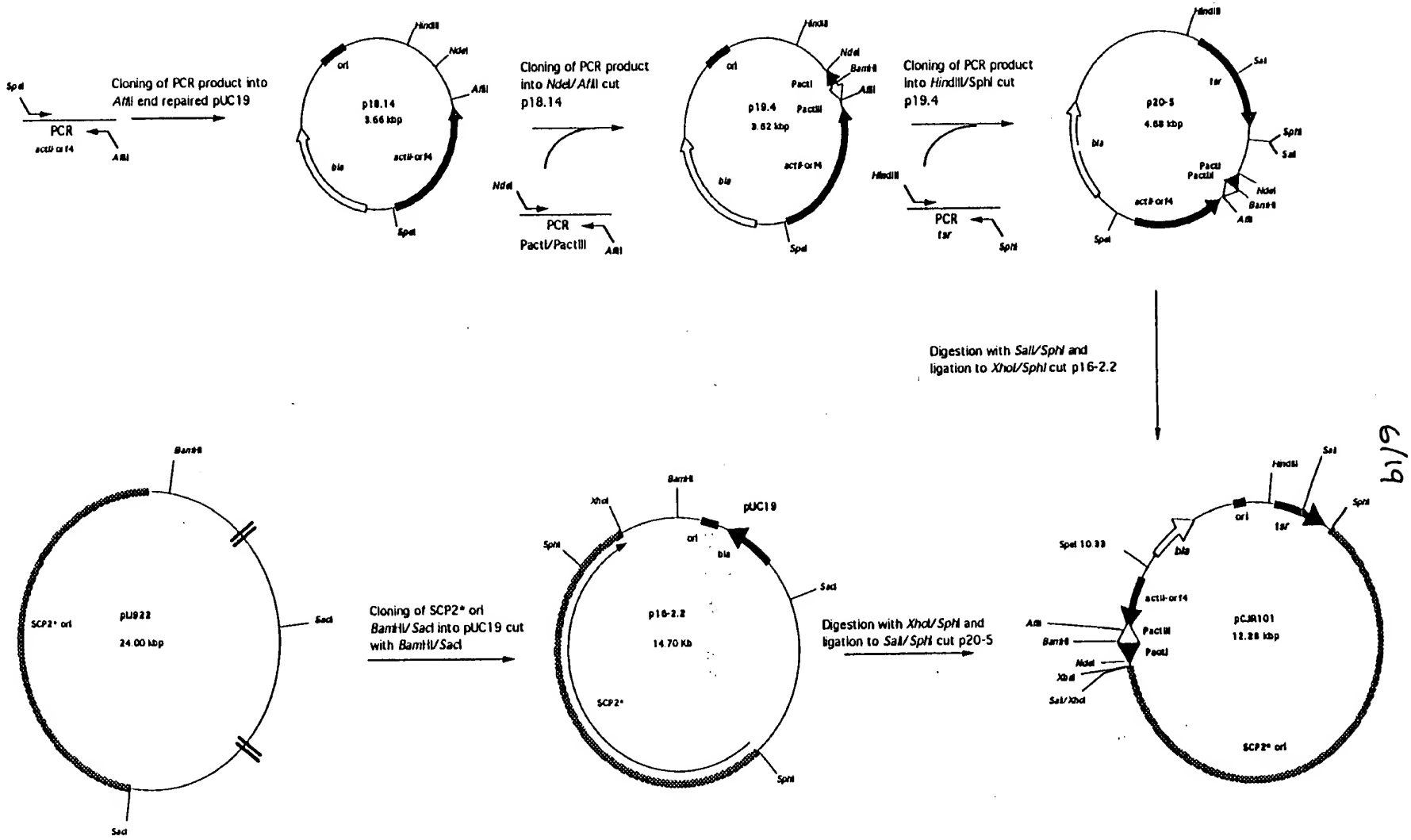

Cloning of PCR product into AflII end repaired pUC19
PCR
actII-orf4
AflII
p18.14
3.66 kbp
Cloning of PCR product into NdeI/AflII cut p18.14
NdeI
PCR
PactI/PactIII
p19.4
3.62 kbp
Cloning of PCR product into HindIII/SphI cut p19.4
HindIII
PCR
tsr
SphI
p20-5
4.68 kbp
Digestion with SalI/SphI and ligation to XhoI/SphI cut p16-2.2
pIJ922
24.00 kbp
SCP2* ori
Cloning of SCP2* ori BamHI/SacI into pUC19 cut with BamHI/SacI
pUC19
p16-2.2
14.70 Kb
SCP2*
Digestion with XhoI/SphI and ligation to SalI/SphI cut p20-5
pCJR101
12.28 kbp
SCP2* ori

Figure 5

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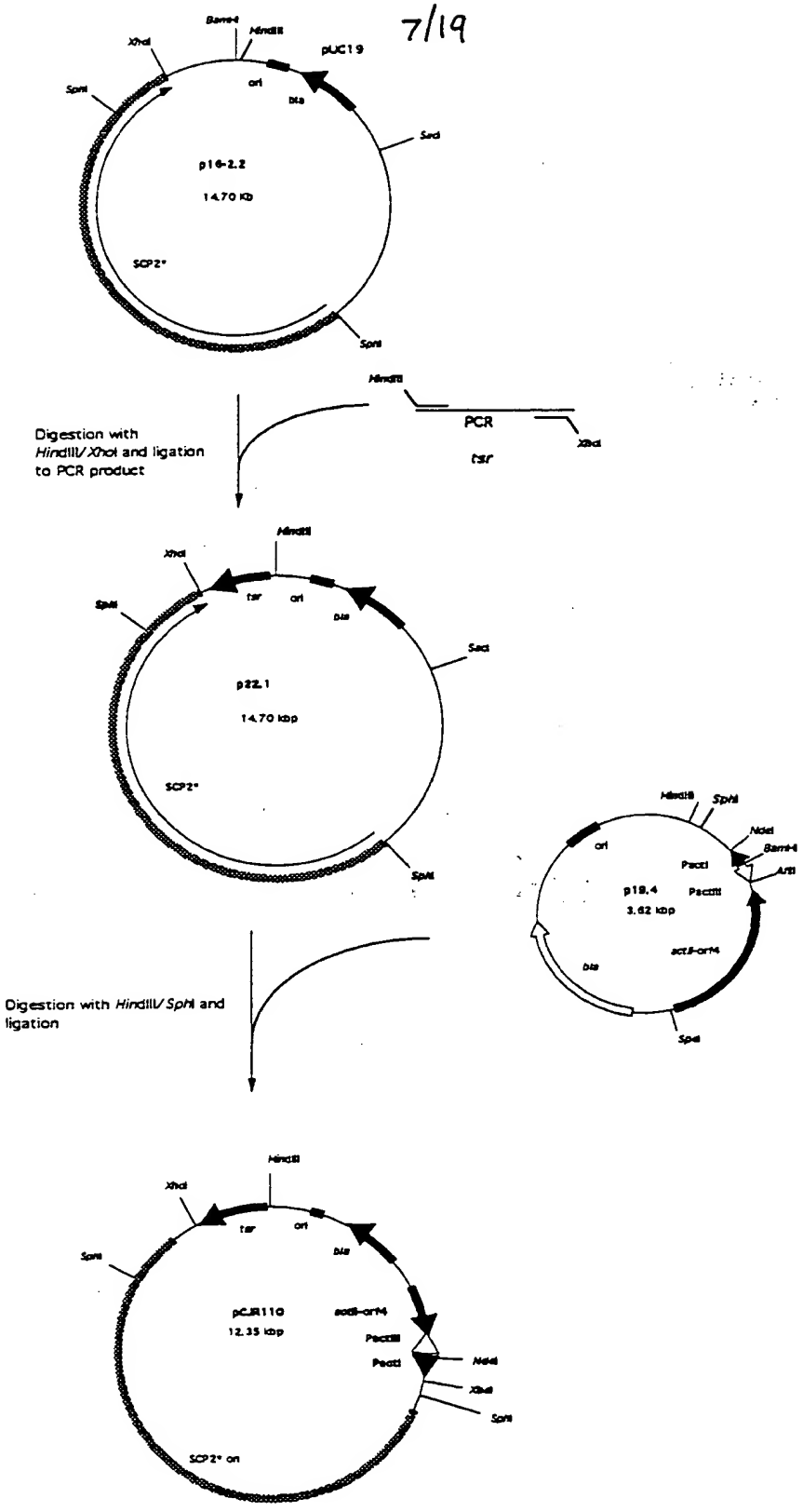
pUC19
p16-2.2
14.70 kb
SCP2*
Digestion with HindIII/XhoI and ligation to PCR product
PCR
tsr
p22.1
14.70 kbp
Digestion with HindIII/SphI and ligation
p18.4
3.62 kbp
pCJR110
12.35 kbp
SCP2* on

Figure 6.

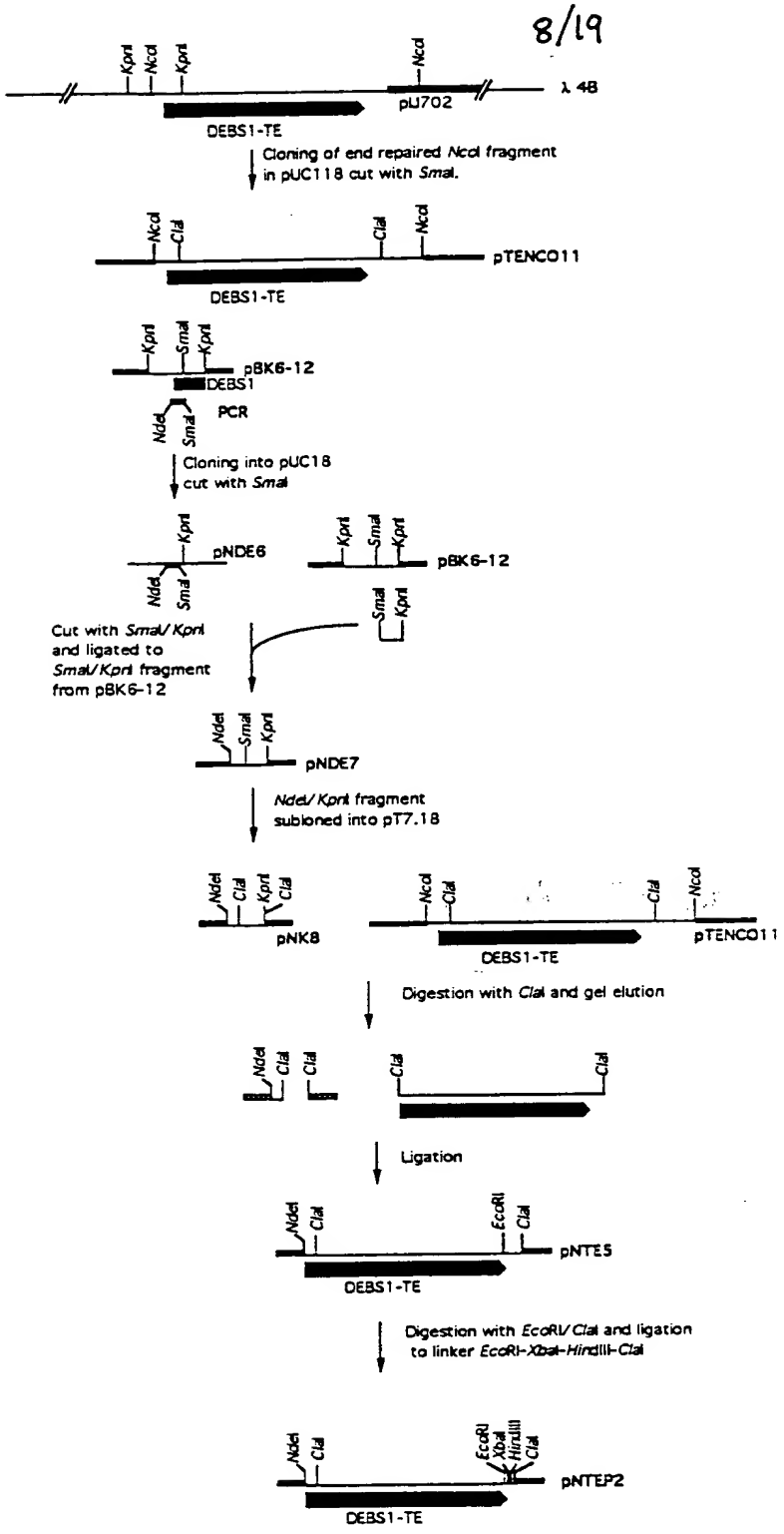
KpnI
NcoI
KpnI
NcoI
λ 48
pU702
DEBS1-TE
Cloning of end repaired NcoI fragment
in pUC118 cut with SmaI.
NcoI
ClaI
ClaI
NcoI
pTENCO11
DEBS1-TE
KpnI
SmaI
KpnI
pBK6-12
DEBS1
NdeI
SmaI
PCR
Cloning into pUC18
cut with SmaI
KpnI
pNDE6
NdeI
SmaI
KpnI
SmaI
KpnI
pBK6-12
Cut with SmaI/KpnI
and ligated to
SmaI/KpnI fragment
from pBK6-12
NdeI
SmaI
KpnI
pNDE7
NdeI/KpnI fragment
subloned into pT7.18
NdeI
ClaI
KpnI
ClaI
pNK8
NcoI
ClaI
ClaI
NcoI
pTENCO11
DEBS1-TE
Digestion with ClaI and gel elution
NdeI
ClaI
ClaI
ClaI
ClaI
Ligation
NdeI
ClaI
EcoRI
ClaI
pNTE5
DEBS1-TE
Digestion with EcoRI/ClaI and ligation
to linker EcoRI-XbaI-HindIII-ClaI
NdeI
ClaI
EcoRI
XbaI
HindIII
ClaI
pNTEP2
DEBS1-TE

Figure 7.

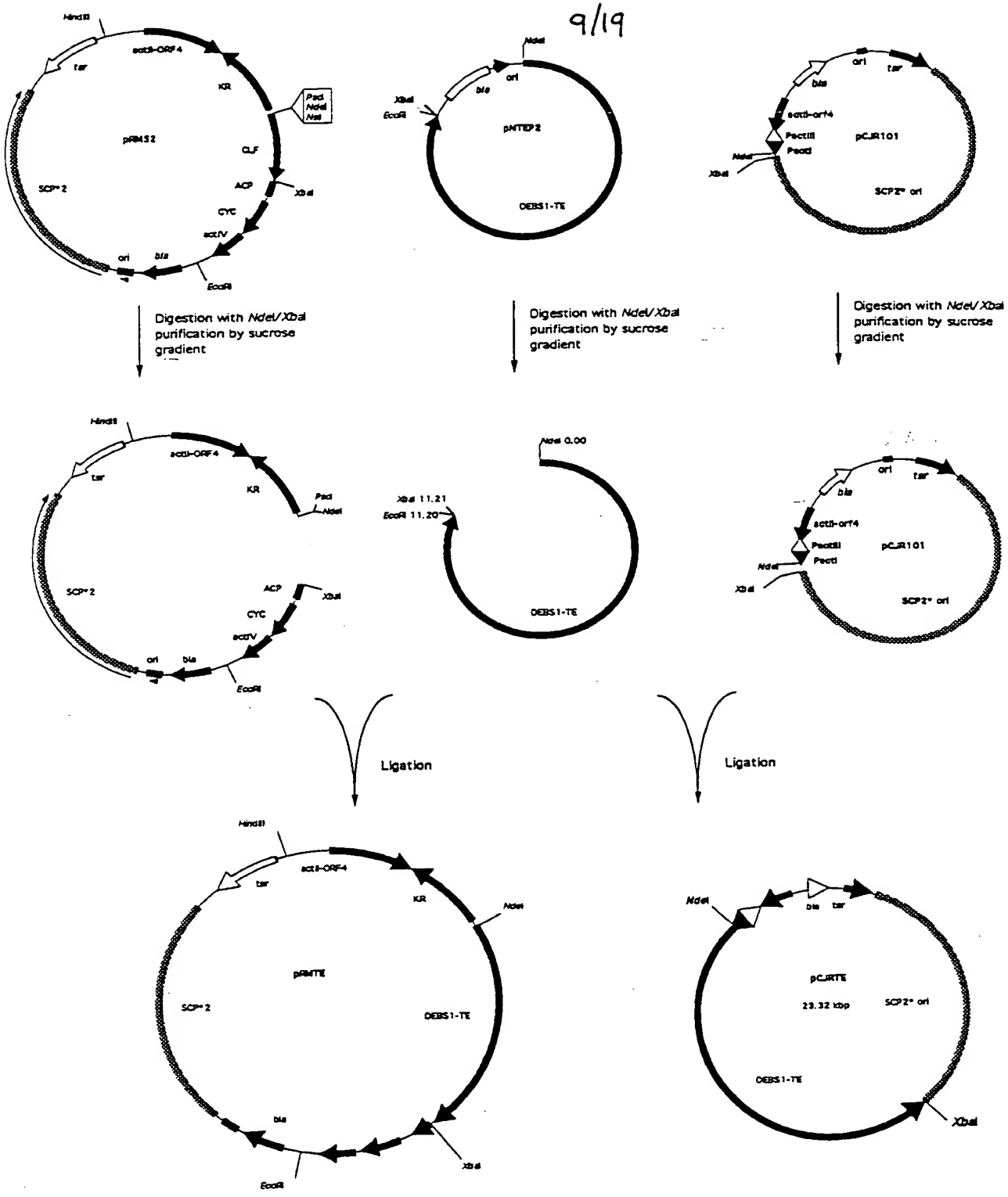
HindIII
actII-ORF4
tsr
KR
PacI
NdeI
NsiI
pRM32
CLF
ACP
XbaI
CYC
actIV
SCP*2
ori
bla
EcoRI
NdeI
ori
XbaI
EcoRI
pNTEP2
DEBS1-TE
ori
tsr
bla
actII-orf4
PacIII
PacI
NdeI
XbaI
pCJR101
SCP2* ori
Digestion with NdeI/XbaI purification by sucrose gradient
Digestion with NdeI/XbaI purification by sucrose gradient
Digestion with NdeI/XbaI purification by sucrose gradient
HindIII
actII-ORF4
tsr
KR
PacI
NdeI
SCP*2
ACP
XbaI
CYC
actIV
ori
bla
EcoRI
NdeI 0.00
XbaI 11.21
EcoRI 11.20
DEBS1-TE
ori
tsr
bla
actII-orf4
PacIII
PacI
NdeI
XbaI
pCJR101
SCP2* ori
Ligation
Ligation
HindIII
actII-ORF4
tsr
KR
NdeI
pRMTE
SCP*2
DEBS1-TE
bla
EcoRI
XbaI
NdeI
bla
tsr
pCJRTE
23.32 kbp
SCP2* ori
DEBS1-TE
XbaI

Figure 8.

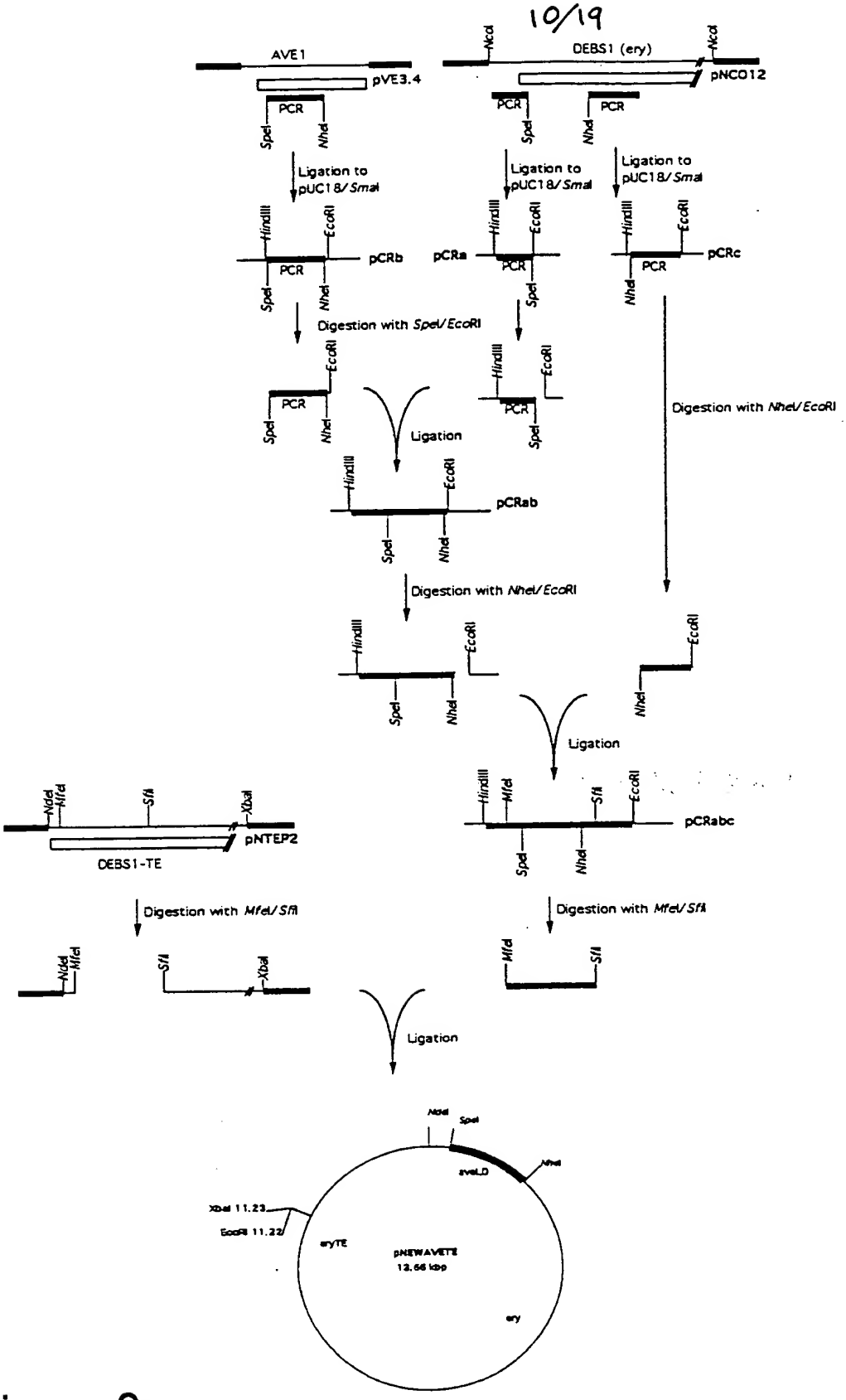
AVE1
pVE3.4
PCR
SpeI
NheI
Ligation to pUC18/SmaI
DEBS1 (ery)
NcoI
pNCO12
pCRb
pCRa
pCRc
HindIII
EcoRI
Digestion with SpeI/EcoRI
Ligation
pCRab
Digestion with NheI/EcoRI
pCRabc
MfeI
SfiI
XbaI
NdeI
pNTEP2
DEBS1-TE
Digestion with MfeI/SfiI
pNEWAVETE
12.66 kbp
aveLD
eryTE
ery
XbaI 11.23
EcoRI 11.22

Figure 9a.

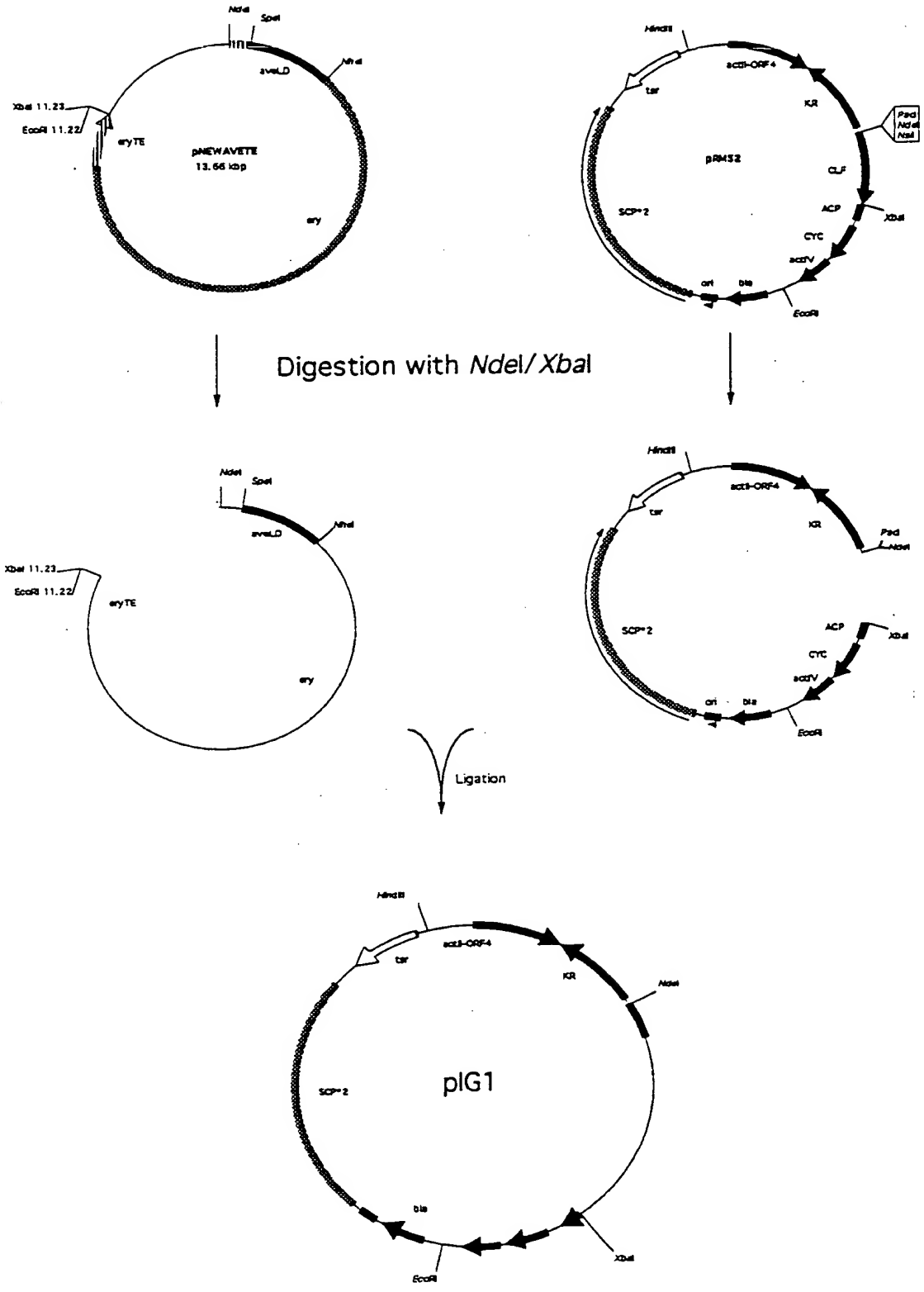
NdeI
SpeI
XbaI 11.23
EcoRI 11.22
eryTE
pNEWAVETE
13.66 kbp
ery
HindIII
tsr
actI-ORF4
KR
PstI
NdeI
NheI
pRMS2
CLF
SCP*2
ACP
XbaI
CYC
actV
ori
bla
EcoRI
Digestion with NdeI/XbaI
Ligation
pIG1

Figure 9b.

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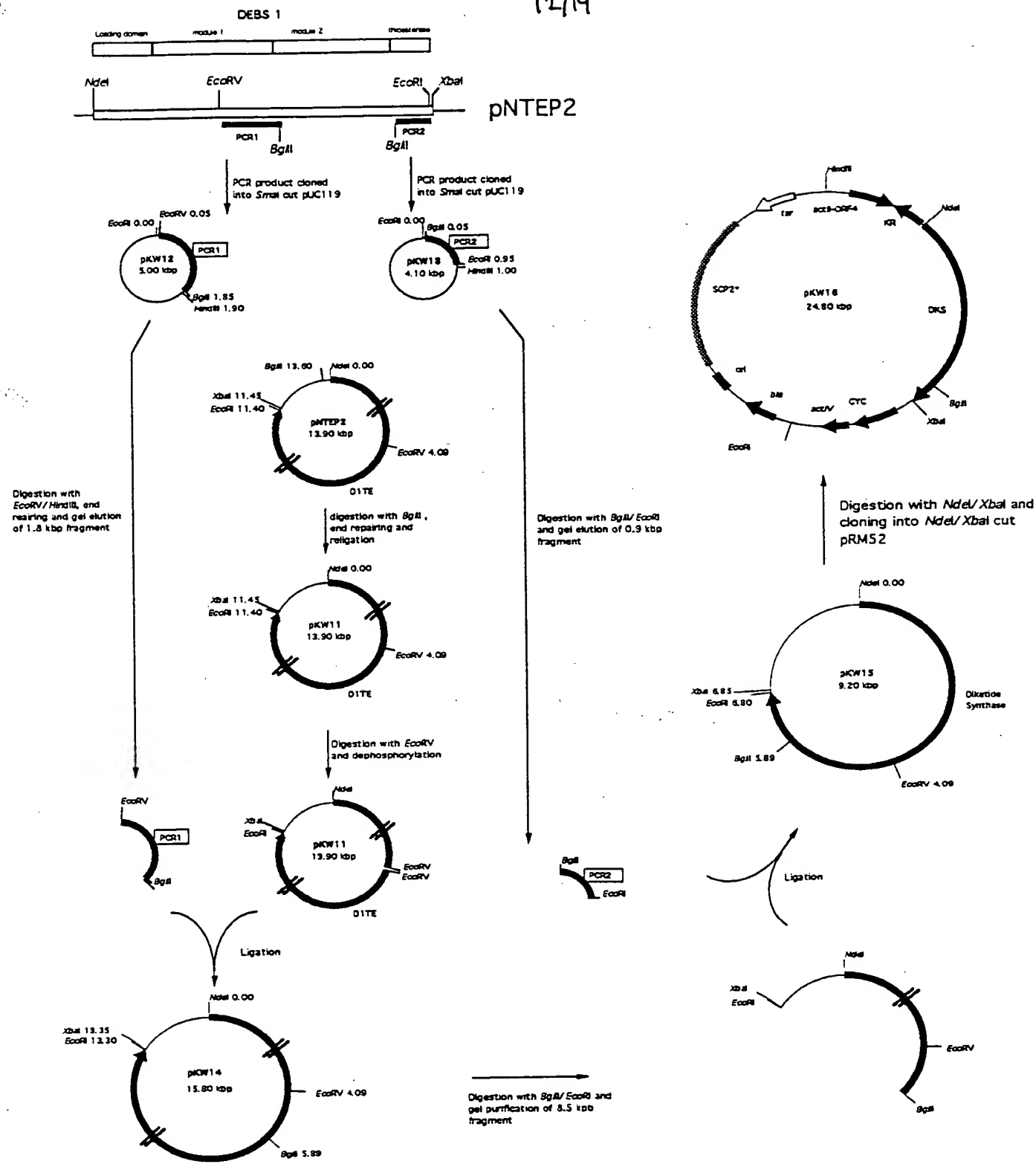

Figure 10.

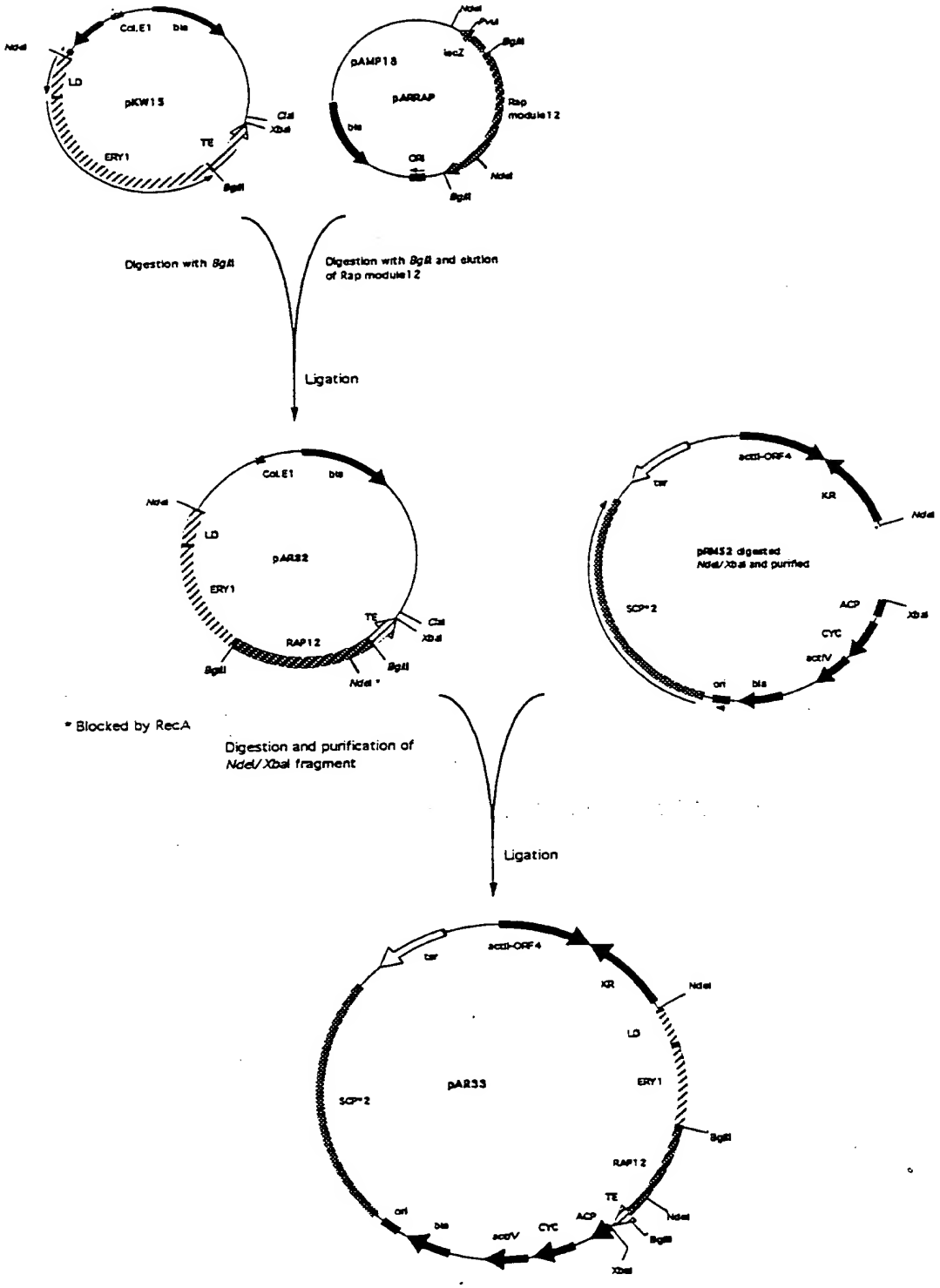
pKW15
pAMP18
pARRAP
Rap module12
Digestion with BglII
Digestion with BglII and elution of Rap module12
Ligation
pAR32
pRM32 digested NdeI/XbaI and purified
* Blocked by RecA
Digestion and purification of NdeI/XbaI fragment
Ligation
pAR33

Figure 11.

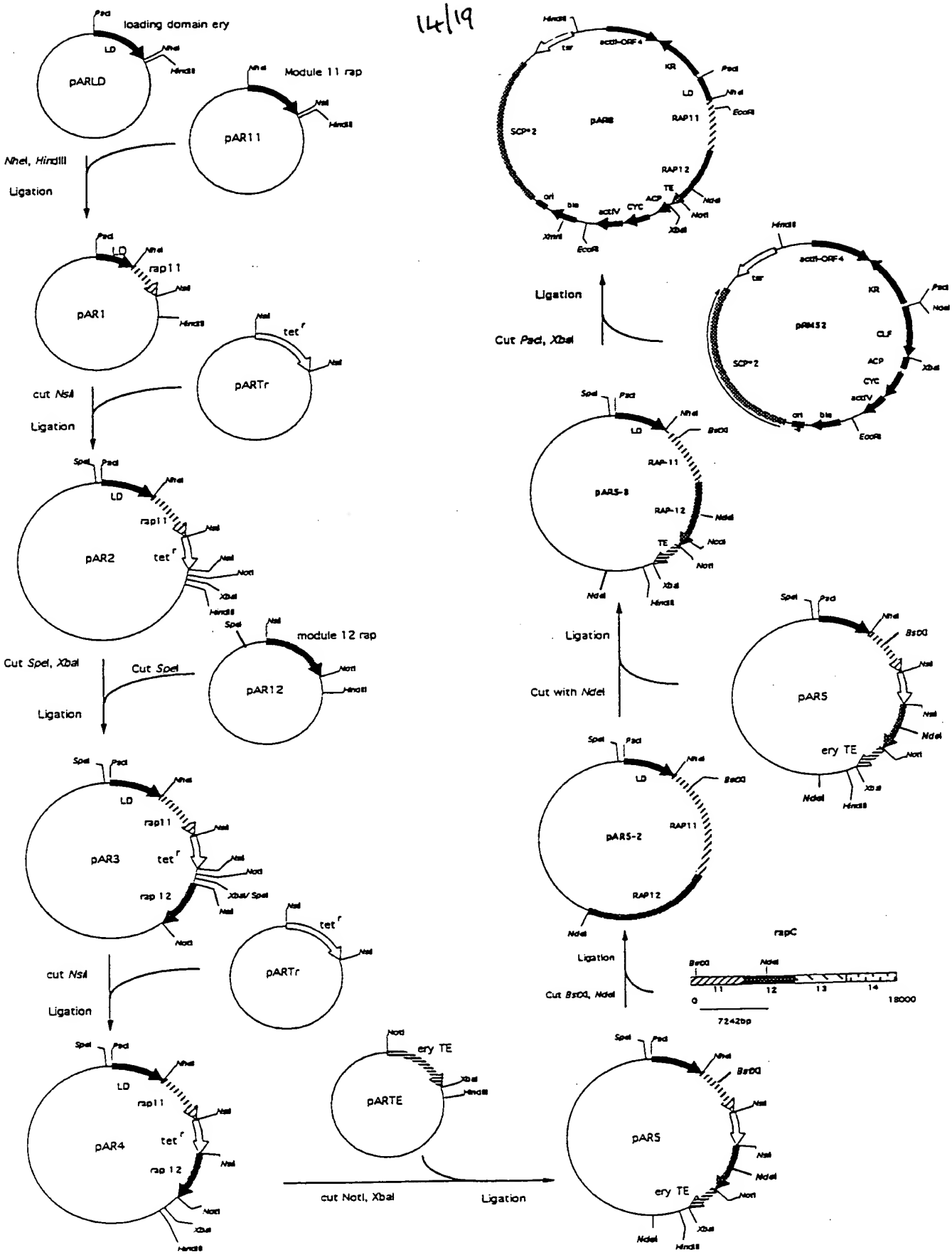
loading domain ery
pARLD
Module 11 rap
pAR11
cut NheI, HindIII
Ligation
pAR1
rap11
tet r
pARTr
cut NsiI
Ligation
pAR2
rap11
tet r
module 12 rap
pAR12
Cut SpeI, XbaI
Cut SpeI
Ligation
pAR3
rap11
tet r
rap 12
pARTr
cut NsiI
Ligation
pAR4
rap11
tet r
rap 12
ery TE
pARTE
cut NotI, XbaI
Ligation
Ligation
Cut PacI, XbaI
Ligation
Cut with NdeI
pAR5
ery TE
pAR5-2
Ligation
Cut BstXI, NdeI
rapC
72420bp
pAR5
ery TE

Figure 12.

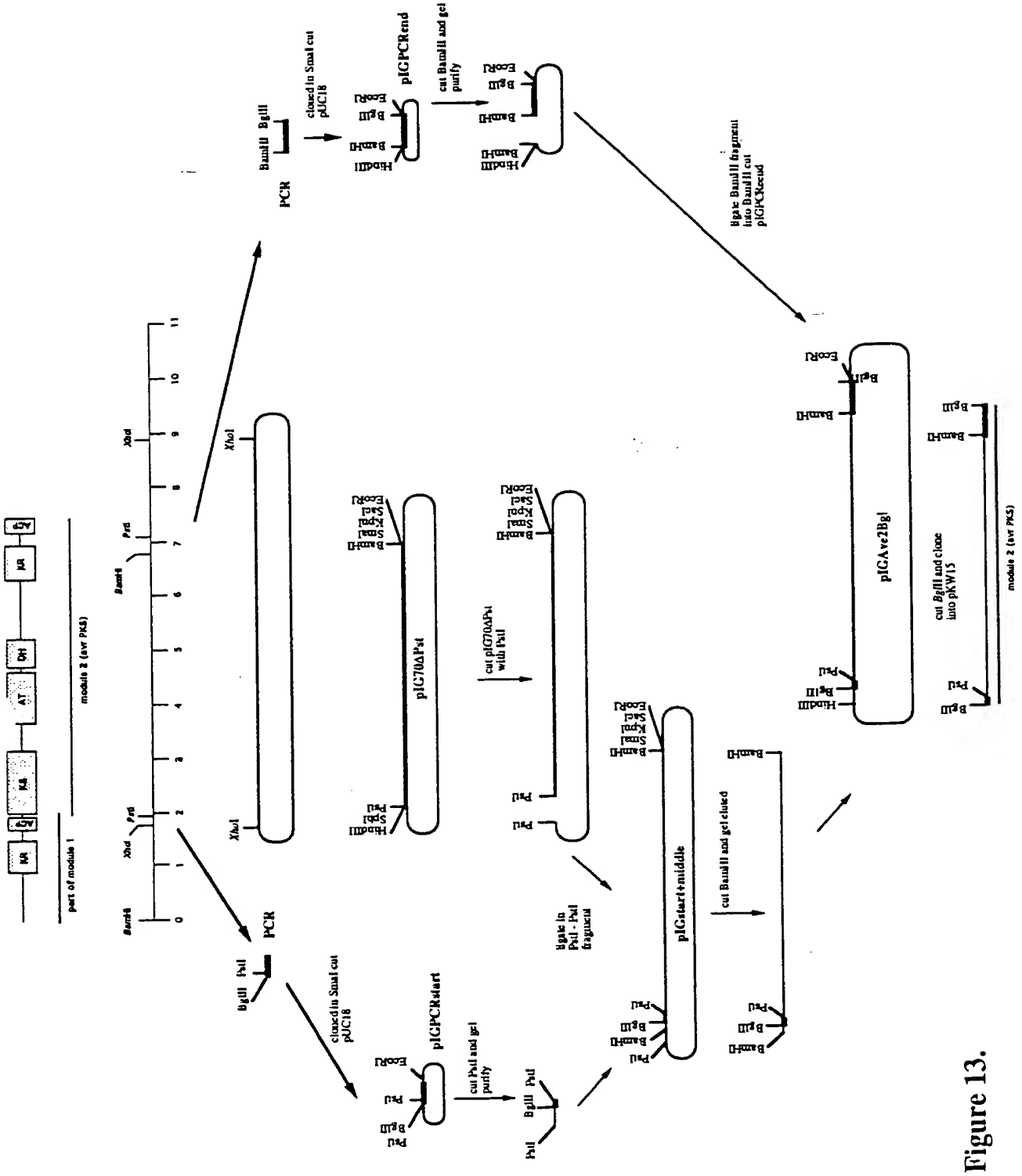
part of module 1
module 2 (avr PKS)
KR
DH
AT
KS
ACP
BamHI
XbaI
PstI
BamH1
Xba1
Pst
0
1
2
3
4
5
6
7
8
9
10
11
PCR
BglII PstI
cloned in SmaI cut pUC18
pIGPCRstart
EcoRI
PstI
BglII
cut PstI and gel purify
pIG70ΔPst
HindIII
SphI
PstI
cut pIG70ΔPst with PstI
pIGstart+middle
BamHI
SmaI
SstI
KpnI
EcoRI
ligate in PstI - PstI fragment
cut BamHI and gel eluted
BamHI
BglII
PstI
BamHI BglII
cloned in SmaI cut pUC18
pIGPCRend
HindIII
BamHI
BglII
EcoRI
cut BamHI and gel purify
ligate BamHI fragment into BamHI cut pIGPCRend
pIGAve2Bgl
HindIII
BglII
PstI
EcoRI
cut BglII and clone into pKW15
module 2 (avr PKS)

Figure 13.

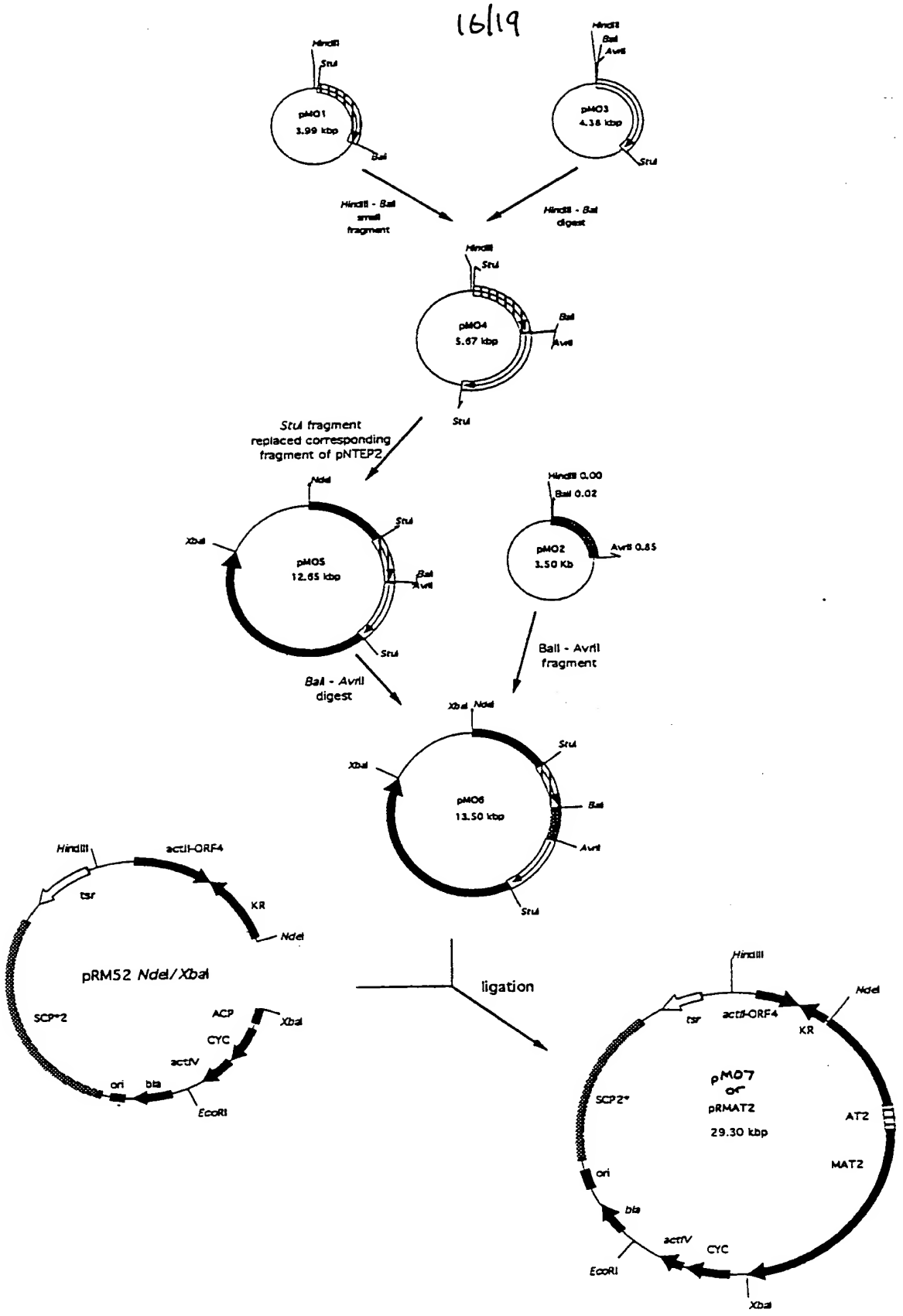
HindIII
StuI
pMO1
3.99 kbp
BalI
pMO3
4.38 kbp
AvrII
HindIII - BalI
small
fragment
HindIII - BalI
digest
pMO4
5.67 kbp
StuI fragment
replaced corresponding
fragment of pNTEP2
NdeI
XbaI
pMO5
12.65 kbp
pMO2
3.50 Kb
HindIII 0.00
BalI 0.02
AvrII 0.85
BalI - AvrII
digest
BalI - AvrII
fragment
pMO6
13.50 kbp
pRM52 NdeI/XbaI
actII-ORF4
tsr
KR
SCP*2
ACP
CYC
actIV
ori
bla
EcoRI
ligation
pMO7
or
pRMAT2
29.30 kbp
AT2
MAT2

Figure 14.

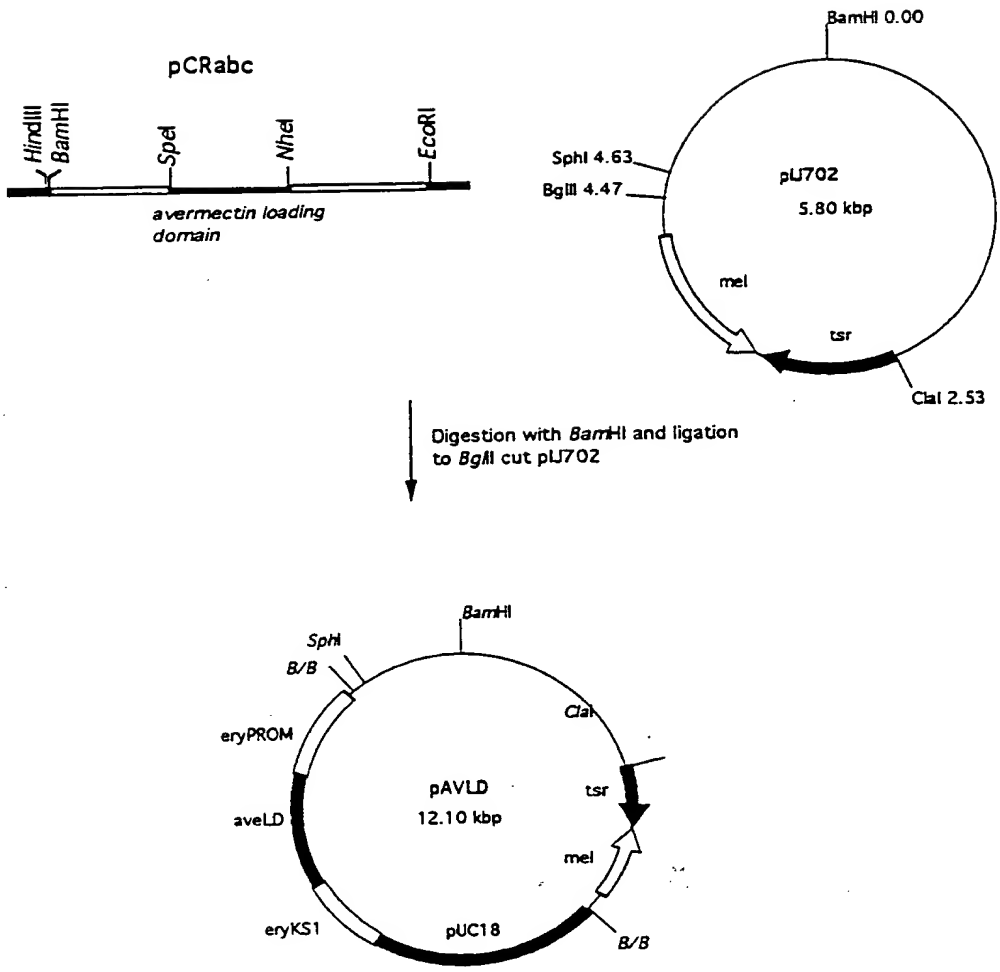
pCRabc
HindIII
BamHI
SpeI
NheI
EcoRI
avermectin loading domain
BamHI 0.00
SphI 4.63
BglII 4.47
pIJ702
5.80 kbp
mel
tsr
ClaI 2.53
Digestion with BamHI and ligation to BglII cut pIJ702
BamHI
SphI
B/B
ClaI
eryPROM
pAVLD
12.10 kbp
tsr
aveLD
mel
eryKS1
pUC18
B/B

Figure 15.

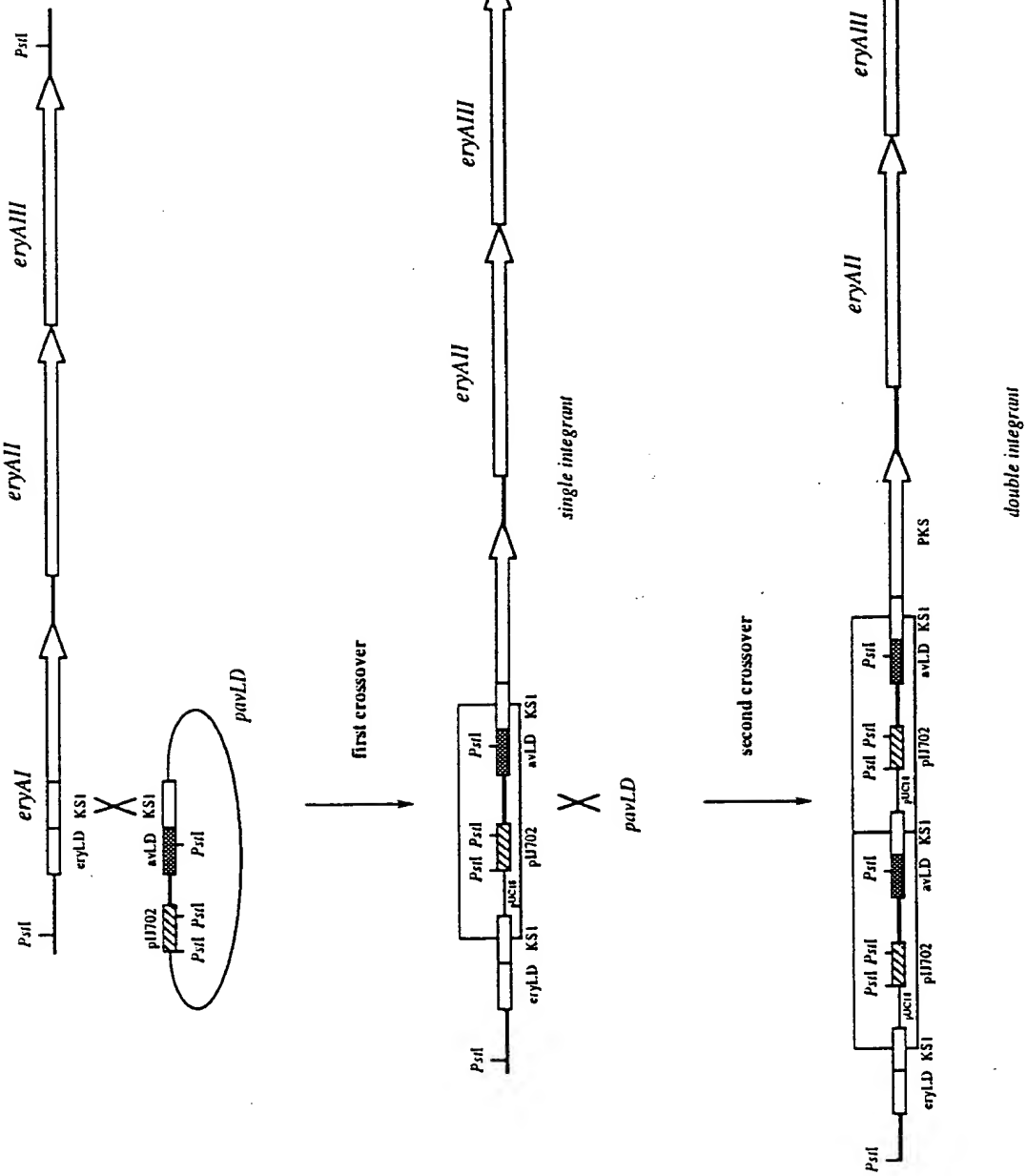
eryAI
eryAII
eryAIII
PstI
eryLD KS1
avLD KS1
pIJ702
pavLD
first crossover
single integrant
second crossover
double integrant
PKS

Figure 16.

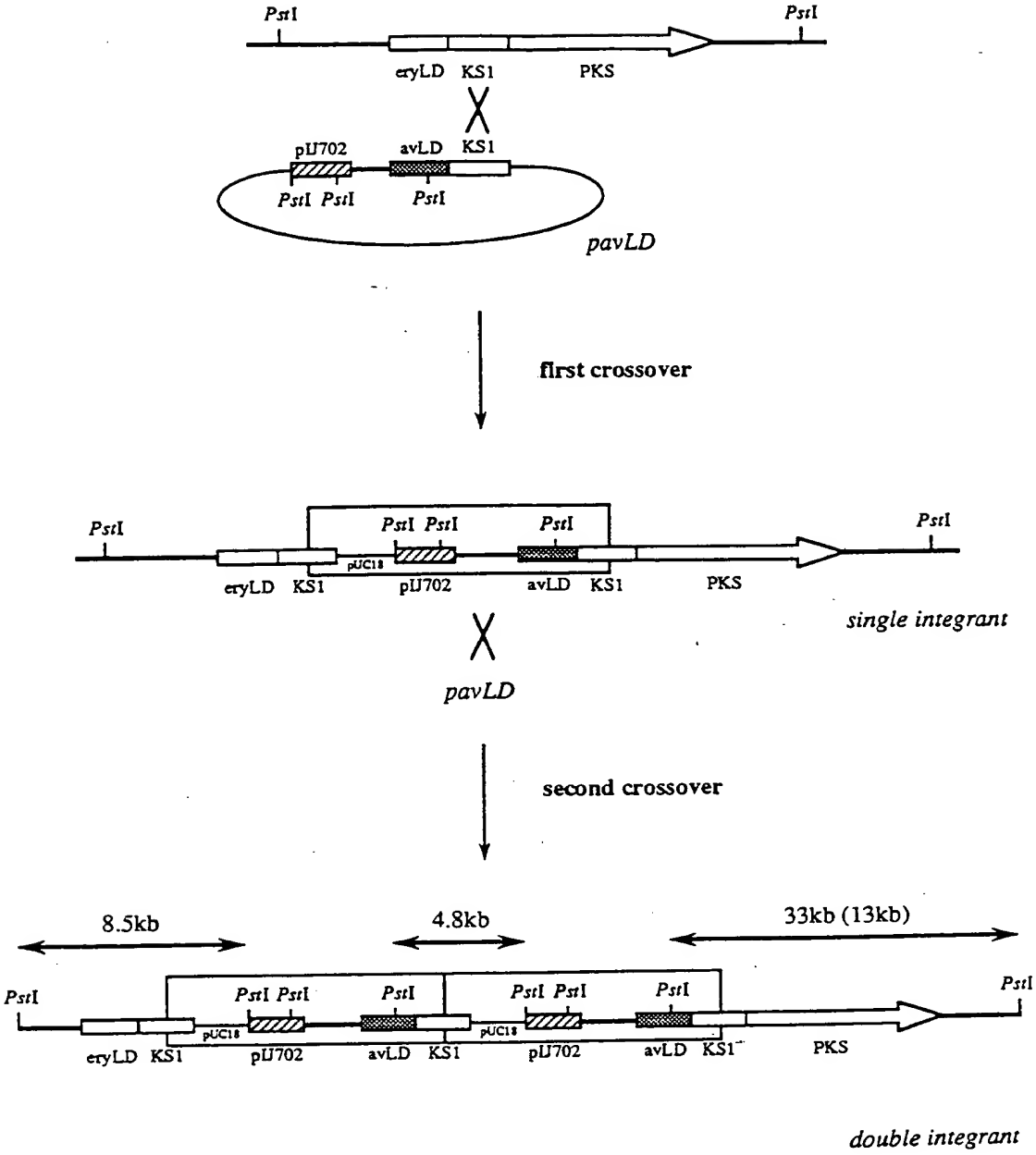
PstI
PstI
eryLD KS1 PKS
pIJ702 avLD KS1
PstI PstI PstI
pavLD
first crossover
PstI
PstI PstI PstI
PstI
pUC18
eryLD KS1 pIJ702 avLD KS1 PKS
single integrant
pavLD
second crossover
8.5kb
4.8kb
33kb (13kb)
PstI
PstI PstI PstI PstI PstI PstI
PstI
pUC18
pUC18
eryLD KS1 pIJ702 avLD KS1 pIJ702 avLD KS1 PKS
double integrant

Figure 17.

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97 10962.3